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(54) Title: TYPE C-LIKE HUMAN RETROVIRUS LINKED TO MULTIPLE SCLEROSIS (MS)

(57) Abstract

The present invention relates to a type C-like human retrovirus. In nested PCR analysis using high stringency PCR conditions and primer pairs and probes used for HIV-I or HTLV-I detection, no genomic sequences are detectable; in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 or p24 or the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind. When performed on purified retrovirus-containing material, reverse transcriptase activity is detectable. A cell culture which additionally is infected with a herpes group virus which enhances the production of the retrovirus has been deposited. Within the scope of the present invention is diagnostic agents comprising genomic fragments of the retrovirus which can be used as nucleotide probes in PCR and diagnostic agents comprising retroviral antigens capable of binding antibodies specific for the retrovirus which can be used for the diagnosis of multiple sclerosis as well as a method for obtaining a protective immunity against multiple sclerosis comprising administering a vaccine against Epstein-Barr virus.

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TYPE C-LIKE HUMAN RETROVIRUS LINKED TO MULTIPLE SCLEROSIS (MS)

Multiple sclerosis

Multiple sclerosis (MS) is a neurological disease of the central nervous system in which smaller or larger ulcerations and/or scars can be found in the central nervous system. In these ulcerations and scars, nerve conduction is more or less - sometimes totally - damaged, because of demyelination of the nerve sheaths. Clinically, multiple sclerosis is highly variable and the rate of progression varies to a high degree from patient to patient (McFarlin and McFarland, 1982).

The disease is associated with clinical symptoms such as sensory, visual and motor dysfunction because of lesions in the nervous system as mentioned above caused by the breakdown of the myelin sheaths. The lesions can be ranging from 1 mm to several centimeters. Clinical diagnosis of the disease can be made by electrophysiologic evaluation, magnetic resonance (MR) and cerebral spinal fluid examination (CSF) (McFarlin and McFarland, 1982). So far, no specific diagnostic test is available and the diagnosis is based on clinical and pathological criteria. The diagnosis may be based on clinical symptoms as mentioned above, that is sensory, visual and motor dysfunction. Futhermore, the diagnose may be based on pathology. The documentation of lesions that have occured on more than one occasion and at more than one site, and which are not explained by other mechanisms is considered as a definitive diagnosis (McFarlin and McFarland, 1982).

The early stage of the disease is manifested by remissions with complete or nearly complete return of normal neurological functions, whereas in the later stages of MS, neurologic dysfunctions progress with only partly or no return of normal neurological functions. In the acute forms of MS no remissions with complete return to normal neurological

functions occurs and the process progresses rapidly over a few weeks or months.

In a mild form of MS also seen, only a few neurological dysfunctions followed by complete recovery is observed and the patients may remain relatively asymptomatic for years thereafter.

Finally, autopsy has indicated that subclinical forms of the disease may exist in which demyelination occurs without resulting in symptoms as those described above.

The possible involvement of retrovirus and Epstein-Barr virus in multiple sclerosis

Tropical spastic paraparesis (TSP) is a chronic progressive myelopathy associated with HTLV-I infection. The disease affects females more often than males and has many similarities to multiple sclerosis (MS) (Poser, 1989). On the basis of epidemiological observations, it has been suggested that MS is associated with an environmental agent (Martyn, 1991). Similarity between MS and TSP has led to the hypothesis that a retrovirus could be involved in MS.

In 1985 antibodies against human retroviruses were claimed to occur more often in MS patients than in controls (Koprowski et al., 1985), but it has not been possible to confirm these findings (Hauser et al., 1986). In 1989 the same group claimed that sequences of HTLV-I could be found more often in MS patients than in controls (Reddy et al., 1989), but these results have not been confirmed either (Prayoonwiwat et al., 1991).

The conclusion from a symposium held in Copenhagen in 1988 on Retrovirus in Multiple Sclerosis and Related Diseases was that multiple sclerosis might be associated with retrovirus and more research should be undertaken to explore this possibility (Haahr, 1992).

Isolation of a leptomeningeal cell line containing retrovirus from a patient with MS (Perron et al., 1989) has been reported in 1989, and in 1991 the same group has reported retroviral particles associated with positive reverse transcriptase activity in mononuclear cells from patients with MS (Perron et al., 1991).

The present inventors have analyzed long-term cultures of cerebrospinal fluid cells and long-term peripheral blood mononuclear cell cultures from MS patients, patients with other neurological diseases and healthy controls for growth characteristics, cell morphology, reverse transcriptase and 2',5'-oligoadenylate synthetase activities. None of these parameters differed between the groups (Höllsberg et al., 1989).

In another study (Sommerlund et al., 1991), multinucleated giant macrophages in long-term cultures derived from MS patients and healthy controls were compared. No differences could be found. Electron microscopy (EM) was performed on these cultures, but no signs of retrovirus-like particles were observed.

Previously, retrovirus-like particles have been found in blood lymphocyte cultures established from a patient with multiple sclerosis (Haahr et al., 1991). The cells were initially stimulated with phytohaemagglutinin (PHA) and cultured with medium containing interleukin-2 (IL-2), and the established cell line was an IL-2-dependent T-cell line. These cells can only be kept in culture for 1-3 months. Because of this, only marginal amounts of these retrovirus-like particles could be produced, and further characterization of this putative virus could not be performed.

EBV

Transactivation of retrovirus by EBV (Ohtsuki et al., 1990) and other viruses of the herpes virus group (Skolnik et al., 1988) is a well known phenomenon.

For several years it has been known that depending on techniques and antigens used, 99-100% of patients with MS have antibodies against EBV, in contrast to controls where only 86-95% have antibodies against this virus (Sumaya et al., 1980; Bray et al., 1983; Larsen et al., 1985; Sumaya et al., 1985). These studies have been confirmed by the present inventors, but it is still unknown whether EBV infection is a prerequisite for development of MS or whether the 100% EBV seropositivity is a consequence of the MS disease.

In a case-control study of MS patients, a strong positive correlation with a previous story of infectious mononucleosis was found, indicating older age at exposure to EBV (Operskalski et al., 1989). In another study, patients with infectious mononucleosis were matched with an MS registry, and a higher risk for MS to occur subsequently to infectious mononucleosis was found (Lindberg et al., 1991).

Epidemiology

If MS is caused by an agent similar to HTLV-I, one could imagine that this agent had the same epidemiology as HTLV-I. Therefore, the intrafamilial epidemiology of MS was compared with the known intrafamilial epidemiology of infections with HTLV-I (Haahr et al., 1991). The study did not reveal any common intrafamilial pattern of MS and HTLV-I infections. It was concluded that if MS is associated with a specific "MS virus", it is hardly one with the same epidemiology as HTLV-I, perhaps because MS could be a multifactorial disease only developing if various factors coincide in the same person.

The cause of MS is thus unknown even though a wide number of possibilities including different types of retroviruses have been proposed as the cause of MS. However, no definite proof of such an association between a retrovirus and MS has been presented until now.

Brief description of the invention

Recently, a B-lymphoblastoid cell line (LCL) of polyclonal origin was isolated from a 30-year-old male patient with a chronic progressive myelopathy, clinically resembling multiple sclerosis as described in Example 1. The LCL expresses Epstein-Barr virus (EBV) encoded proteins and on EM the LCL was shown to produce both EBV particles and retrovirus-like particles spontaneously. The LCL was negative for human retrovirus (HIV-1 and HTLV-I) sequences by a polymerase chain reaction (PCR) assay using high stringency PCR conditions as described in Example 9. Furthermore, as described in Example 4, the patient was seronegative to these retroviruses as well as HTLV-II and HIV-2. It is contemplated that the LCL is double-infected with EBV and a hitherto uncharacterized human retrovirus. The EM pictures was examined by Dr. Hans Gelderblom, who confirmed the observation. The spontaneous production of EBV is remarkable because this usually takes place only in cell lines induced by exogenous factors like halogenated pyrimidines or infection by a second virus. Thus, human retroviruses have been found to induce EBV in cell lines (Lai et al., 1989).

The cell line (MS-1533) is now established and growing continuously in the inventors' laboratory, producing 0.5-1 billion cells per week. Production of virus has been followed by transmission EM. Production of retrovirus-like particles takes place for a period of at least 5-6 months after establishing the cultures from frozen ampoules. Production of intact EBV particles only takes place initially,

later on defect EBV is produced. Both viruses have been purified by a two-fold sucrose gradient ultracentrifugation as described in Example 5A. Verification of this purification is performed by negative staining EM as described in Example 8.

Immunoelectron microscopy gives the possibility of capturing virus particles so that they are seen in greater numbers than what would be seen by normal negative staining procedures where the virus solution is spread directly onto the grids. Furthermore, a more "clean" picture is seen since cell organelles or other small cell parts are washed away. In the material shown in Figure 2b, the antibody was a mouse monoclonal antibody to HTLV-1 gp46 which had been shown to react with MS 1533 in Western blotting assays. The viral solution was cell supernatant double purified by sucrose gradient centrifugation.

Initially it was not possible to obtain a positive reverse transcriptase assay. However, in the purified material, the reverse transcriptase assay is clearly positive in the gradients where EM shows retrovirus-like particles as described in Example 6. Electrophoresis on material from these gradients has been performed and in material from the gradient where retrovirus is seen, one antigen has been found; this antigen reacts with an antibody raised against an HTLV-I antigen (see Example 7 for details). The fact that polyclonal HTLV-I antibodies detected only one of several antigens clearly indicates that it is not a question of HTLV-I.

The synopsis of these results has led to the tantalizing hypothesis that the etiological agent for development of MS and MS-like diseases is a hitherto uncharacterized retrovirus, but development of neurologic disease is related to or even dependent on a delayed EBV infection. This dual infection hypothesis has been analyzed and was found to be in

accordance with the most consistent epidemiological characteristics of MS (Martyn, 1991).

The potential role of cytokines as pathogenic factors

Even if retrovirus and EBV would be found to be the etiological agents in MS, it would most probably still be necessary to explain the pathogenic process in MS as an immunological reaction. Here cytokines may play an essential role.

A spontaneous higher production of IL-6 has been found in mononuclear cells from MS patients compared with cells from controls (Sommerlund et al., 1991). A spontaneous production of IL-6, β-IFN and TNF has been found in the cell line MS 1533 (see Example 3).

IL-6 is a cytokine which influences both T and B lymphocytes, playing a special role for antibody production. This could explain the polyclonal B-cell activation in MS patients and the high amount of antibody in cerebrospinal fluid and serum. TNF has been found toxic to oligodendrocytes and it can cause demyelination (Selmaj et al., 1988). Evidence of intrathecal synthesis of TNF in MS has been found, and the level of TNF in cerebrospinal fluid correlates with the severity and progression of the disease (Sharief et al., 1992).

Retrovirus

Reverse transcriptase

Reverse transcriptase (RT), a crucial marker of retrovirus, has been found in cells from MS patients by one group (Perron et al., 1989; Perron et al., 1991). The present inventors have been searching for RT activity for an extended period without significant results, since only a little more than borderline activity could be detected.

With the large number of cells available from the cell line MS 1533, it has been possible to purify and concentrate supernatants to a degree where RT activity is clearly seen. A further characterization of the reverse transcriptase and of the optimum conditions for its detection is currently being performed.

Antigen characterization

MS 1533 has been characterized antigenetically, using several polyclonal as well as monoclonal antibodies raised against the various HTLV-I antigens. The antibodies were from several different sources.

Using Western Blotting techniques on purified viral particles obtained by double-gradient centrifuged supernatants, it has been found that the retrovirus is not HTLV-I; this is described in detail in Example 7. Two of the most prominent HTLV-I antigens could not be identified. On the other hand, a positive signal has been identified as described in detail in Example 7 using a monoclonal antibody against a specific antigen, which signal cannot be caused by background or unspecific binding. This distinct protein shows the expected size in molecular weight, indicating the presence of a retroviral related protein.

Sequence analysis

The fact that one of the antigens has been positively identified facilitates the further characterization of the retrovirus at the nucleic acid level. Oligonucleotide primers, their sequence either being deduced from the antigen or based on known retroviral "consensus" sequences, is utilized for PCR analysis of cDNA synthesized from RNA isolated from the LCL as described in Example 9. The synthesized DNA fragments may be used both as probes for the relevant retroviral sequences in the LCL genome and as templates for a preliminary sequence analysis.

The oligonucleotides as well as the characterized PCR products can be used as probes for the assessment of viral expression levels, either by quantitative PCR analysis or by various blotting techniques. The concomitant use of oligonucleotides specific for either EBV or the retrovirus will help elucidating the double infection and may be used as a diagnostic tool.

Antibody studies

The purification and concentration of antigens as described in Example 5 make it possible to use Western Blotting techniques for antibody studies and to develop an ELISA for both the viruses involved in MS, thus greatly facilitating the processing of larger numbers of patient sera.

Establishing new cell lines

Mononuclear peripheral blood cells are continuously being grown, and new cell cultures are being established from both patients with chronic neurological diseases and healthy controls in order to look for retrovirus-like particles in the cells by EM. The cells are grown without activating factors but with and without IL-2. In this way, it is possible to establish both T-cell lines and B-lymphoblastoid cell lines.

EBV

In order to further elucidate the association of EBV with MS, it is desirable to determine the occurrence of IgA antibodies in serum from MS patients and controls. In patients with nasopharyngeal carcinoma, a disease which is suggested to be associated with EBV, high titers of IgA have been found in serum (Henle et al., 1976).

If possible, CSF and blood from patients with MS will be examined as well as controls for cells secreting antibodies against EBV, thereby examining whether EBV antibodies are produced within the CNS in MS. As mentioned above, oligonucleotides specific for EBV may also be used in order to elucidate the infection in cells with EBV, for example in plaques.

To clarify previous studies (Operskalski et al., 1989; Lindberg et al., 1991) showing a higher risk for MS to occur subsequent to infectious mononucleosis, information has been obtained about 4000 persons who showed a positive Paul-Bunnell reaction 20-25 years ago, confirming an ongoing infectious mononucleosis. These 4000 persons have been searched in the Danish MS Registry in which information about more than 95% of all Danish MS cases are registered. At the same time a control group of 14000 persons suspected for having infectious mononucleosis but showing a negative Paul-Bunnell reaction, indicating that they did not have an acute infection with EBV at the time of examination was searched in the Danish MS Registry.

In the group of 4000 persons with a positive Paul-Bunnell (PB) reaction, 11 persons later developed multiple sclerosis, whereas only 14 out of the 14000 persons with a negative Paul-Bunnell reaction later developed MS. None of the 4000 persons with a confirmed infectious mononucleosis had signs of MS before this infection. This is in contrast to 9 persons from the control group, who had a diagnosed MS before beeing tested with a PB reaction. The study showed a 2.75 times higher risk of developing MS subsequent to infectious mononucleosis.

Cytokines

Studies on the production of IL-6 in MS patients and controls <u>in vivo</u> and <u>in vitro</u> as described in Example 3 are continued.

The production of cytokines in LCL (MS 1533) is further evaluated, and new cell lines established is analyzed for these cytokines using quantitative PCR analysis.

Detailed description of the invention

According to the present invention, a new retrovirus has now been found which is associated with MS, and which, therefore, opens up a wide range of possibilities for reliable diagnosis of MS. Also, the production of the retrovirus has been found to co-exist with production of Epstein-Barr virus for which reason it is justified to assume that the retrovirus is activated by Epstein-Barr virus; this gives rise to the provision of a strategy for the development of a vaccine treatment for MS, such as will appear from the following.

The retrovirus of the invention is found in cells from patients suffering from MS, including early stages of MS. Thus, one cell culture producing the retrovirus is a lymphoblastoid B cell culture from a patient with a chronic progressive neurological disease with a marked spasticity of the legs. The cell culture was established from blood mononuclear cells without stimulation with PHA and without interleukin-2 in the medium. The first weeks, the culture appeared as a culture of adherent macrophages with few non-adherent cells in the medium. After several weeks, small clones of larger lymphocytes appeared in the culture. In the beginning, these small clones were adhering to the macrophages. This cell culture is described in greater detail in Example 1 and in the following.

The retrovirus appears, in transmission electron microscopy performed as described in Examples 2 or 8, to be a type C-like retrovirus, and a number of tests as reported in the following have confirmed non-identity between the retrovirus and a number of known retroviruses.

Thus, in one aspect, the invention relates to a cell culture comprising cells which are infected with a type C-like retrovirus which is present in human patients who have symptoms indicating an early stage of multiple sclerosis, the retrovirus being a retrovirus which can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the cell culture using the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK39, SK70, SK19 and SK31

and in PCR using the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the cell culture,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the cell culture.

In a further aspect, the invention relates to a cell culture comprising cells which are infected with a type C-like human retrovirus, the retrovirus being a retrovirus

which can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

a) in which the retrovirus shows the following negative tests:

A) in nested PCR analysis on the cell culture using high stringency PCR conditions, the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK30, SK70, SK19 and SK31

and in PCR analysis using high stringency PCR conditions, the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the cell culture,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the cell culture,

and the retrovirus showing the following positive tests:

A) in Western blotting performed on retrovirus- containing material purified by sucrose gradient purification or Triton X-114 temperature induced phase separation

or purified by adsorption to antibody-conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable,

B) in reverse transcriptase assay performed on retrovirus-containing material doubble purified on sucrose gradients, revers transcriptase activity is detectable,

c) in PCR analysis using low stringency PCR conditions and using the following primer pairs

477/478, 1898/1899, 1900/1901, 1956/1957 and 2345/2346,

PCR products are detectable.

The above tests, which are described in detail in the examples, confirm that the retrovirus is not identical with known retroviruses.

The appearance of the retrovirus particles in transmission electron microscopy appears from Fig. 1c and 1d. Using negative staining electron microscopy the spherical particles have substantially the appearance as shown in figures 2a and 2b.

The retrovirus of the invention is identified as a type C-like retrovirus in accordance with the description given in Dalgleish et al. 1990, as the retrovirus possesses various morphologic features characteristic to retroviruses of type C. No visible projections from the outer cell membrane is observed, only an ill defined central core structure is present and no identifiable intracytoplasmic precursor forms are observed, all of these features being key features in the identification of type C viruses. Furthermore,

the size of the retrovirus being 80-120 nm is in accordance with the morphological features of a type C virus.

The cell culture is preferably one which is capable of actively producing the retrovirus, so that the retrovirus can be produced using the cell culture and then isolated, concentrated and purified, and so that antigens and nucleotide sequences characteristic to the retrovirus can be obtained from the culture. Thereby, the cell culture according to the invention constitutes an important source of diagnostic materials and starting materials for diagnostic materials, such as will appear from the following. It has been found that the culture of the invention actively produces the retrovirus when the cell culture is additionally containing a herpes group virus, in particular Epstein-Barr virus. It is believed that the expression/product of the herpes group virus, such as Epstein-Barr virus enhances the production of the retrovirus; it is well known that such an interaction between different types of viruses can occur. Another herpes group virus which is contemplated to be particularly interesting for enhancing the production of the retrovirus is human herpes virus-6 (Schonnebeck et al., 1991).

The cell culture may be a mammalian cell culture, in particular a simian or human cell culture.

Examples of cells useful or contemplated to be useful for establishing the cell culture are lymphoblastoid cells or myelomonocytary cells, in particular lymphoblastoid B cells. An interesting cell culture is one in which the cells are capable of growing without adherence to surfaces.

The cell culture according to the invention may be established on the basis of cells from body fluids, such as blood, or from tissue samples, from patients suffering from MS as established by clinical or definitive diagnosis or from MS-like diseases such as chronic progessive myelo-

pathies, said patients being infected with the retrovirus, and in particular such patients whose serum does not contain antibodies against other retroviruses. The cell culture may be established using conventional techniques, such as described in Example 1, and the presence of the retrovirus in the cell cultures is confirmed by transmission electron microscopy and/or negative staining electron microscopy and the various tests as described herein, in particular the tests described above and PCR or hybridization tests as described below.

A cell culture of the invention, named MS1533, has been deposited with ECACC, European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts, SP4 0JG, UK, on 4 October, 1991 and was given the provisional deposit number V 91100401. Because of a low number of cells in the initial deposit, cultivation of cells was made at the ECACC. A new deposit of MS1533 was made on 8 April, 1992 and was given the provisional deposit number V 92040805. It will be understood that whenever the provisional deposit number V 91100401 is referred to in the present specification and claims, the culture is derived from the same source as the culture given the provisional deposit number V 92040805 and that the latter number can therefore be substituted for the former number.

The cell culture of the invention can also be defined as a cell culture infected with the retrovirus with which said deposited cell cultures is infected or with a retrovirus which is identical therewith except for genetic variations which are commonly found in retroviruses and which do not change the above-defined properties of the retrovirus. It is well known that all living organisms are subject to genetic variation and mutation in most cases without losing neither its identifying characteristica nor its characteristic functions, and hence, it is evident that not only the specific retrovirus of the deposited cell culture, but also a range of functionally and morphologically identical retroviruses with slight genetic variation will be useful

as diagnostic material and starting material for producing diagnostic materials such as disclosed herein, for which reason the invention, when characterized on the basis of the deposited cell culture, also comprises cell cultures infected with such variations of the retrovirus, provided that the retrovirus passes the above-mentioned tests.

Another way of characterizing the range of cell cultures according to the invention is by reference to the fact that they contain genomic fragments which can also be found by PCR in blood samples from diagnosed multiple sclerosis patients, but not in blood samples from healthy persons.

By the term "healthy person" is meant a person who is not infected with the retrovirus of the invention.

A further way of characterizing the range of cell cultures according to the invention is by reference to the fact that they contain antigens capable of binding antibodies which are present in sera from diagnosed multiple sclerosis patients, such antigens being antigens which do not bind antibodies present in sera from healthy persons.

The invention also relates to the purified retrovirus in the form of whole retrovirus or fragments thereof. Such purified retrovirus or fragments thereof may be obtained by known methods, e.g. by rupturing the cells of a cell culture as defined above and concentrating/purifying the retrovirus or fragments thereof, e.g. by affinity chromatography, such as antibody affinity chromatography using immobilized antibodies specific to the retrovirus. The production of such antibodies is described in the following. Alternatively, the retrovirus containing material can be purified by the methods described in Example 5 (sucrose gradient purification, Triton X-114 temperature induced phase separation or purification by adsorption to antibody-conjugated microbeads). The purified retrovirus or fragments thereof are useful, e.g. as starting material for

sequencing purposes, as antigenic/immunogenic material for the production of further antibodies, and as diagnostic agents or starting materials for diagnostic agents, e.g. diagnostic agents as discussed below.

The invention also relates to an antigen or epitope derived from, produced by, or induced by the retrovirus with which the above-identified cell culture is infected, or derived from the above-mentioned purified retrovirus, the antigen showing the following negative tests:

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the antigen or epitope,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the antigen or epitope,

D) in immunoassays, antibodies directed against HIV-I, and HIV-II do not bind to the antigen or epitope,

the antigen or epitope being obtainable by subjecting cell fragments and/or medium from a cell culture as discussed above or purified retrovirus as described above to gel electrophoresis, applying serum from a diagnosed multiple sclerosis patient to the resulting gel and visualizing bound antibody by means of labelled anti-human antibody, comparing the visualized gel bands with a similar preparation made using sera from a number of healthy persons, identifying the bands which are antibody-bound in the preparation using the serum from a diagnosed patient and which are not bound in the preparations using sera from the healthy persons, isolating such bands containing the antigen or epitope from corresponding gel electrophoresis, and optionally extracting the antigen or epitope from the bands and purifying the antigen or epitope. It will be understood that once an identification as described above has been

performed, that is, once the bands in the gel characteristic of the antigen or epitope have been identified, further productions of the antigen or epitope can be performed simply by securing identity with the bands first found by the use of the sequences of test criteria described above, e.g. by repeating the gel electrophoresis under exactly the same conditions and isolating the antigen or epitope from the bands having the position identified to be the position of the characteristic antigens or epitopes.

In a further aspect, the antigens or epitopes of the invention further show the following positive test:

A) in Western blotting performed on retrovirus- containing material purified by sucrose gradient purification or Triton X-114 temperature induced phase separation or purified by adsorption to antibody-conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable.

The antigens or epitopes of the invention, characteristic to the cell culture and/or the retrovirus, are very valuable diagnostic agents for the diagnosis of MS in any infected stage, such as active stage or early stage or prestage or subclinic stage or latent infection, all of which are defined below, in which infected stage antibodies against the antigen are produced in the patient. Thereby, a specific and easy diagnostic test for MS is made avaialable. Likewise, anti-idiotypic antibodies as defined below may be valuable diagnostic agents for the diagnosis of MS in any of the below defined infective stages in patients.

In connection with the diagnostic and other aspects of the present invention, the following explanations/definitions of the various stages of MS are relevant:

With the term "prestage" is meant a stage of infection with the retrovirus in a patient, in which the clinical and/or pathological symptoms have not yet developed but in which the immune system has been presented to the retrovirus and in which the retrovirus is activated and will give rise to MS over an unknown period of time.

With the term "early stages of infection" is meant a stage of infection with the retrovirus in a patient, in which the retrovirus is activated and has given rise to clinical symptoms comprising sensory and/or visual and/or motor dysfunction and/or pathological symptoms comprising lesions but where remission to complete or nearly complete return of normal neurological functions occurs. In this stage, lesions of variable dimensions have been observed.

With the term "diagnosed multiple sclerosis" is meant a stage of infection with the retrovirus in a patient, in which the clinical symptoms comprising sensory, visual and/or motor dysfunction and pathological symptoms comprising lesions of the nervous system have been diagnosed, by a definitive diagnosis.

With the term "subclinic infection" is meant an infection with the retrovirus in a patient, in which the retrovirus gives rise to only pathological signs such as demyelination shown under autopsy, but where no clinical symptoms characteristic to MS can be observed or was observed.

With the term "latent infection" is meant an infection in a patient with the retrovirus, in which the retrovirus will not give rise to any clinical and/or pathological symptoms indicating MS or an early stage thereof unless activated, possible by an infection with a herpes group virus such as Epstein-Barr virus.

The invention also relates to a method of diagnosing multiple sclerosis or a prestage thereof, a latent infection or

a subclinical infection with the retrovirus of the culture according to the invention expressing the antibody as defined above, comprising contacting a sample of body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent comprising an antigen or epitope of the invention or an anti-idiotypic antibody as defined below, and determining the presence of any antibody from the sample bound to the antigen, epitope, or anti-idiotypic antibody.

The diagnostic test may be performed using a diagnostic agent which comprises an antigen or epitope of the invention or an anti-idiotypic antibody as described below, bound to a carrier or support as described below. Any antibodies from the sample binding to the antigen, epitope, or anti-idiotypic antibody may be detected using a secondary antibody capable of binding to the first bound antibody and provided with a label as described below.

The substance used as label may be selected from any substance which is in itself detectable or which may be reacted with another substance to produce a detectable product. Thus, the label may be selected from radioactive isotopes, enzymes, chromophores, fluorescent or chemiluminescent substances, and complexing agents.

Examples of enzymes useful as labels are β-galactosidase, urease, glucose oxidase, carbonic anhydrase, peroxidases (e.g. horseradish peroxidase), phosphatases (e.g. alkaline or acid phosphatase), glucose-6-phosphate dehydrogenase and ribonuclease.

Enzymes are not in themselves detectable, but must be combined with a substrate to catalyze a reaction the end product of which is detectable. Thus, a substrate may be added to the reaction mixture resulting in a coloured, fluorescent or chemiluminescent product or in a colour change or in a change in the intensity of the colour,

fluorescence or chemiluminescence. Examples of substrates which are useful in the present method as substrates for the enzymes mentioned above are H_2O_2, p-nitrophenylphosphate, lactose, urea, β-D-glucose, CO_2, RNA, starch, or malate. The substrate may be combined with, e.g. a chromophore which is either a donor or acceptor.

Fluorescent substances which may be used as labels for the detection of the components as used according to the of invention may be 4-methylumbelliferyl-phosphate, 4-methylumbelliferyl-D-galactopyranoside, and 3-(p-hydroxyphenyl) propionic acid. These substances may be detected by means of a fluorescence spectrophotometer. Chemiluminescent substances which may be peroxidase/eosin/EDTA, isoluminol/-EDTA/H_2O_2 and a substrate therefor.

Chromophores may be o-phenylenediamine or similar compounds. These substances may be detected by means of a spectrophotometer.

Radioactive isotopes may be any detectable and in a laboratory acceptable isotope, e.g. ^{125}I, ^{131}I, ^{3}H, ^{32}P, ^{35}S or ^{14}C. The radioactivity may be measured in a γ-counter or a scintillation counter or by radioautography followed by densitometry.

Complexing agents may be Protein A, Protein G (which forms a complex with immunoglobulins), biotin (which forms a complex with avidin and streptavidin), and lectin (which forms a complex with carbohydrate determinants, e.g. receptors). In this case, the complex is not in itself directly detectable, necessitating labelling of the substance with which the complexing agent forms a complex. The marking may be performed with any of the labelling substances described above.

As indicated above, this type of diagnostic agent normally comprises the antigen or epitope or the anti-idiotypic

antibody bound to a carrier or support so that appropriate washing or other treatments as appropriate may be performed without risk of appreciable loss of the bound antigen, epitope, or anti-idiotypic antibody. The carrier or support is normally solid, and the antigen, epitope or anti-idiotypic antibody is bound to the carrier or support by any suitable mode of binding, such as hydrogen bonding, hydrophobic bonding, van der Waals' forces, covalent bonding, etc.

In an embodiment of the invention, the antigen, epitope or anti-idiotypic antibody of the invention may be indirectly coupled to a solid support via a bridging compound or "linker". The linker, which is designed to link the solid support and the antigen, the epitope may be hydrazide, Protein A, glutaraldehyde, carbodiimide, or lysine.

The solid support employed is e.g. a polymer or it may be a matrix coated with a polymer. The matrix may be of any suitable solid material, e.g. glass, paper or plastic. The polymer may be a plastic, cellulose such as specially treated paper, nitrocellulose paper or cyanogenbromide-activated paper, silicone, silica, or a polysaccharide such as agarose or dextran. Examples of suitable plastics are latex, a polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylacetate and any suitable copolymer thereof. Examples of silicone polymers include siloxane.

The solid support may be in the form of a tray, a plate such as a mitrotiter plate, e.g. a thin layer or, preferably, strip, film, threads, solid particles such as beads, including Protein A-coated bacteria, or paper.

The invention also relate to a monoclonal or polyclonal antibody which binds to an antigen or epitope as defined above and also to an anti-idiotypic antibody as described below. A most useful type of antibody is a monoclonal antibody; however, also a polyclonal antibody may be of

great importance provided it shows a sufficient selectivity, which may be obtained, e.g., by means of known absorption methods.

The term "antibody" refers to a substance which is produced by a mammal or more precisely a cell of mammalian origin belonging to the immune system as a response to exposure to the polypeptides of the invention.

The variant domain of an antibody is composed of variable and constant sequences. The variant part of the domain is called the idiotype of the antibody. This part of the antibody is responsible for the interaction with the antigen, the antigen binding.

The idiotypic structure is antigenic and can thus give rise to specific antibodies directed against the idiotypic structure. Production of such anti-idiotypic antibody has been done in mice. The antibodies raised against the idiotype, the anti-idiotypic antibodies, may mimic the structure of the original antigen and therefore may function as the original antigen to raise antibodies reactive with the original antigen. This approach may be advantageous as it circumvents the problem associated with the characterization and synthesis of the important immunogenic parts of the antigen in question. This is most important in the case of conformational epitopes, which might otherwise be difficult to identify. The present invention therefore also relates to an anti-idiotypic antibody which is directed against the site of an antibody which binds the antigen or the epitope according to the invention.

The antibodies of the present invention may be produced by a method which comprises administering in an immunogenic form at least a part of the antigen or epitope of the invention or an anti-idiotypic antibody as defined above to obtain cells producing antibodies reactive with said polypeptide and isolating the antibody containing material

from the organism or the cells. The methods of producing antibodies of the invention will be explained further below.

In a further aspect, the invention relates to a diagnostic agent which comprises an antibody as defined above, preferably a monoclonal antibody. The diagnostic agent may comprise the antibody coupled to a carrier or support. Alternatively, the diagnostic agent may be in the form of a test kit comprising in a container an antibody as defined above. The diagnostic agent may be used in the diagnosis of MS or a prestage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to the invention expressing the antigen or epitope of the invention, comprising contacting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent comprising an antibody as defined above, and determining the presence of any antigen or epitope from the sample binding to the antibody.

The diagnostic agent may be one which is suited for use in an agglutination assay in which solid particles to which the antibody is coupled agglutinate in the presence of an antigen or an epitope of the invention in the sample subjected to testing. In this type of testing, no labelling of antibody is necessary. For most uses it is, however, preferred that the antibody is bound to a carrier or support, using, e.g., the techniques described above in connection with diagnostic agents based on an antigen, epitope, or anti-idiotypic antibody, and that the binding of antigen or epitope from the sample to the diagnostic agent is detected using a secondary antibody which is capable of binding to the thus bound antigen or epitope, the second antibody being provided with a label for the detection of bound secondary antibody. The substance used as label may be selected from any substance which is in itself detectable or which may be reacted with another substance to

produce a detectable product. Thus, the label may be selected from radioactive isotopes, enzymes, chromophores, fluorescent or chemiluminescent substances, and complexing agents, all of which are described in greater detail above.

The antibody of the invention may be used in an assay for the identification and/or quantification of at least a form and/or a part of the antigen or epitope of the invention present in a sample. The identification and/or quantification performed by the use according to the present invention may be any identification and/or quantification involving the antigen or epitope of the invention. The identification and/or quantification may be performed for both a scientific, a clinical and an industrial purpose. As will be further described below, it is especially important in clinical routine to identify or quantify antigens or epitopes of the invention.

Furthermore, the antigen or epitope or anti-idiotypic antibody may be used in an assay for the identification and/or quantification of antibodies reactive with the antigen or epitope of the invention and being present in a sample, e.g. as defined above. This assay may be carried out by use of a method comprising contacting the sample with the antigen or epitope of the invention and detecting the presence of bound antibody resulting from said contacting and correlating the result with a reference value.

In one preferred embodiment of the invention it is preferred that the antibody used in the method of the invention is a monoclonal antibody as this generally provides a higher precision and accuracy of the assay, at the same time possibly requiring less time to perform. Furthermore, a mixture of two or more different monoclonal antibodies may be employed as this may increase the detection limit and sensitivity of the test. The monoclonal antibody may be obtained by the method described below. Antibodies possess-

ing high avidity such as poloclonal antibodies may be selected for catching techniques.

The antibody used in the present method is preferably in substantially pure form (purified according to suitable techniques or by the methods of the invention, see below) in order to improve the precision and/or accuracy of the assays of the invention.

Another field of the invention is a method for producing an antibody which binds to the antigen or the epitope of the invention, which comprises immunizing an animal with the antigen or epitope or an anti-idiotypic antibody or an antigen or epitope produced by cultivating cells harbouring a plasmid which contains and is capable of expressing a nucleotide sequence as described below which codes for a polypeptide which has the properties of the antigen or epitope as described above, or by synthetically producing a polypeptide having an amino acid sequence derived from the nucleotide sequence of the retrovirus of the invention, whereby cells producing an antibody specific for the antigen is obtained and the antibody is isolated from the animal or the cells.

The antibody is preferably a monospecific antibody. The monospecific antibody may be prepared by injecting a suitable animal with a substantially pure preparation of the polypeptide of the invention followed by one or more booster injections at suitable intervals (e.g. one or two weeks to a month) up to four or five months before the first bleeding. The established immunization schedule is continued, and the animals are bled about one week after each booster immunization, and antibody is isolated from the serum in a suitable manner (cf. e.g. Harboe and Ingild, 1973).

For purposes not requiring a high assay specificity, the antibody may be a polyclonal antibody. Polyclonal anti-

bodies may be obtained, e.g. as described in Harboe and Ingild, see above. More specifically, when polyclonal antibodies are to be obtained, the compound comprising an antigen or epitope of the invention or an anti-idiotype antibody as described above is prepared and preferably after addition of a suitable adjuvant, such as Freund's incomplete or complete adjuvant, injected into an animal. The animals are bled regularly, for instance at weekly intervals, and the blood obtained is separated into an antibody containing serum fraction, and optionally said fraction is subjected to further conventional procedures for antibody purification, and/or procedures involving use of purified compounds comprising an antigen or epitope of the invention or idio-typic antibody as described above.

In another preferred embodiment, monoclonal antibodies are obtained. The monoclonal antibody may be raised against or directed substantially against an antigen or epitope of the invention as described above or an anti-idiotypic antibody as described above. The monoclonal antibody may be produced by conventional techniques (e.g. as described by Köhler and Milstein, 1975), e.g. by use of a hybridoma cell line, or by clones or subclones thereof or by cells carrying genetic information from the hybridoma cell line coding for said monoclonal antibody. The monoclonal antibody may be produced by fusing cells producing the monoclonal antibody with cells of a suitable cell line, and selecting and cloning the resulting hybridoma cells producing said monoclonal antibody. Alternatively, the monoclonal antibody may be produced by immortalizing an unfused cell line producing said monoclonal antibody, subsequently growing the cells in a suitable medium to produce said antibody, and harvesting the monoclonal antibody from the growth medium.

The immunized animal used for the preparation of antibodies of the invention is preferably selected from the group consisting of rabbit, monkey, sheep, goat, mouse, rat, pig, horse and guinea pigs. The cells producing the antibodies

of the invention may be spleen cells, lymph cells or peripheric lymphocytes.

When hybridoma cells are used in the production of antibodies of the invention, these may be grown in vitro or in a body cavity of an animal. The antibody-producing cell is injected into an animal such as a mouse resulting in the formation of an ascites tumour which releases high concentrations of the antibody in the ascites of the animal. Although the animals will also produce normal antibodies, these will only amount to a minor percentage of the monoclonal antibodies which may be purified from ascites by standard purification procedures such as centrifugation, filtration, precipitation, chromatography or a combination thereof.

An example of a suitable manner in which the monoclonal antibody may be produced is as a result of fusing spleen cells from immunized mice (such as Balb/c mice) with myeloma cells using conventional techniques (e.g. as described by Dalchau et al. 1980). The fusions obtained are screened by conventional techniques such as binding assays employing compounds comprising antigen or epitope of the invention or an anti-idiotypic antibody as described above isolated by the above-described methods.

The invention also relates to a nucleic acid having a nucleotide sequence which is characteristic to the above-identified retrovirus. In other words, the nucleotide sequence is one which is distinct from sequences from known retroviruses, and at the same time is indicative of the presence of the above-identified new retrovirus. Such nucleic acids are nucleotide sequences which, when used as a probe on samples of body fluid such as blood samples or tissue samples from a number of healthy persons and blood samples or tissue samples from diagnosed multiple sclerosis patients, respectively, detects nucleotide sequences

from diagnosed multiple sclerosis patients, which can not be detected in samples from the healthy persons.

In other words, the nucleotide sequences serve as a specific marker of the retrovirus of the invention. The nucleotide sequences can be obtained by isolating nucleotide sequences derived from the cell culture described above or the purified retrovirus or fragments thereof as described above by the use of retrovirus-specific nucleotide primers recognizing specific regions of the nucleotide sequences from the above-described retrovirus. The virus-specific nucleotide primers (which also constitute an aspect of the invention) can be developed using on the one hand a cell culture according to the invention and on the other hand primers which are not specific to the present retrovirus, but which contain nucleotide sequences which will recognize retrovirus generally, such as known conserved regions of nucleotide sequences from various retroviruses, using the following strategy: The non-specific, but generally retrovirus-recognizing primers can be used to obtain nucleotide sequences derived from the cell culture according to the invention or the purified retrovirus according to the invention by PCR. In the next stage, the nucleotide sequences thus obtained can be sequenced to identify regions thereof which are distinct from sequences from known retroviruses, and these distinct sequences can then be tested by using them as primers on samples from a number of healthy persons and samples from diagnosed MS patients, respectively, and selecting the sequences which give rise to the attainment of oligonucleotide sequences from diagnosed MS patients, but do not give rise to attainment of oligonucleotide sequences from the healthy persons. Evidently, the sequencing could in principle be excluded, but this would make more testing on samples necessary.

In a specific embodiment, the invention relates to a nucleic acid having a nucleotide sequence (S) obtainable by using a retrovirus-related nucleotide primer recognizing

conserved regions of known retroviruses to obtain nucleotide sequences derived from the cell culture according to the invention or, alternatively, the purified retrovirus according to the invention by PCR, optionally sequencing the nucleic acids obtained to identify sequences which are distinct from sequences from known retroviruses, testing the nucleic acids obtained by PCR or the sequences identified by sequencing by using them as primers on blood samples from a number of healthy persons and blood samples from diagnosed multiple sclerosis patients, respectively, and selecting, as the nucleotide sequence (S), the nucleic acids or sequences which give rise to the attainment of nucleotide sequences from diagnosed multiple sclerosis patients in PCR, but do not give rise to attainment of nucleotide sequences from healthy persons, or using such nucleic acids or sequences as primers for an additional PCR obtainment of nucleic acids from the cell culture according to the invention, performing the testing defined above using such second generation nucleic acids or sequences identified therein as primers and selecting, as the nucleotide sequence (S), the nucleic acids or sequences which give rise to the attainment of nucleotide sequences from diagnosed multiple sclerosis patients in PCR, but do not give rise to attainment of nucleotide sequences from healthy persons.

By the term "nucleotide sequences" is meant any nucleotide sequences of various length, preferably an oligonucleotide sequence. The nucleotide sequence may be either a RNA nucleotide sequence or a DNA nucleotide sequence.

In a specific embodiment, the invention relates to a diagnostic agent comprising a nucleotide probe which is capable of detecting a nucleotide sequence according to the invention.

The invention also relates to a nucleotide probe which is capable of detecting a nucleotide sequence as defined

above. Both the nucleotide sequence and the nucleotide probe are valuable for use in diagnostic agents, such as PCR kits, for diagnosing MS.

Whereever used, the term "probe" describes any nucleotide sequence, preferably an oligonucleotide sequence, which may be used to obtain nucleotide sequences complementary to the probe, e.g. nucleotide sequences suitable for use as primers in PCR or for use as probes in hybridization techniques.

The substance used to label the probe may be selected from any substance which is in itself detectable or which may be reacted with another substance to produce a detectable product. Thus, the label may be selected from radioactive isotopes, enzymes, chromophores, fluorescent or chemiluminescent substances, and complexing agents.

Examples of enzymes useful as labels are β-galactosidase, urease, glucose oxidase, carbonic anhydrase, peroxidases (e.g. horseradish peroxidase), phosphatases (e.g. alkaline or acid phosphatase), glucose-6-phosphate dehydrogenase and ribonuclease.

Radioactive isotopes may be any detectable and in a laboratory acceptable isotope, e.g. ^{125}I, ^{131}I, ^{3}H, ^{32}P, ^{35}S or ^{14}C. The radioactivity may be measured in a γ-counter or a scintillation counter or by radioautography followed by densitometry.

Examples of detection systems based on enzymes are the DIG-system (digoxygenin; Boehringer) and the Tropix-system (Stratagene). Complexing agents used in the detection may be biotin (which forms a complex with avidin and streptavidin). In this case, the complex is not in itself directly detectable, necessitating labelling of the substance with which the complexing agent forms a complex. The mark-

ing may be performed with any of the labelling substances described above.

As indicated above, this type of diagnostic agent normally comprises the labelled probe, which reacts with or detects the presence of complementary sequences in a sample of a body fluid, such as a blood sample, or a tissue sample. Either probe or sample may be bound to a carrier or support, so that appropriate treatments may be performed without risk of appreciable loss of the bound material. The carrier or support is normally solid, and the probe or sample is bound to the carrier or support by any suitable method of binding, such as hydrogen bonding, van der Waal's forces or covalent binding.

In an embodiment of the invention, the labelled probe or sample may be indirectly coupled to a solid support via a bridging compound or a linker. The linker, designed to link the solid support and the labelled probe or sample, may be streptavidin.

The solid support employed is e.g. a polymer or it may be a matrix coated with polymer. The matrix may be of any suitable solid material, e.g. glass, paper or plastic. The polymer may be a nylon or nitrocellulose. Examples of suitable plastics are polystyrene or polyvinylchloride. The solid support may be in the form of a tube, a thin layer or strip, threads, solid particles such as beads or paper.

Thus, the invention also relates to a method of diagnosing multiple sclerosis, an early stage or a pre-stage thereof, a latent infection or a subclinical infection with the retrovirus of the culture defined above, the method comprising subjecting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient to a PCR analysis in which the sample is contacted with a diagnostic agent comprising the nucleotide probe or the nucleotide sequence defined above, and any

detected nucleotide sequence is allowed to be amplified, followed by detection of any amplified target nucleotide sequence. The PCR analysis is a well-established technique described, e.g., in Sambrook, 1990.

Alternative diagnostic methods can be be hybridization as described in standard textbooks in the field. These can be performed e.g. as described in EP 326 395 which describes a method of detecting and identifying a human retrovirus in a human blood or tissue sample comprising (a) amplifying a portion of the human retrovirus env or consensus RNA present in the sample.

In a further aspect, the invention relates to a method of diagnosing multiple sclerosis, an early stage or a pre-stage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to the invention comprising subjecting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient to a PCR analysis in which the sample is contacted with a diagnostic agent according to the invention allowing any nucleotide sequence to be amplified followed by detection of any amplified target nucleotide sequence.

In another aspect, the invention relates to a method of in vitro diagnosing multiple sclerosis, an early stage or a pre-stage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to the invention, optionally combined with a method for detection of an infection with another virus such as a herpes group virus, comprising contacting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent according to the invention comprising a nucleic acid according to the invention and determining the presence of any identical or homologous nucleotide sequences in the sample.

In another aspect, the invention relates to a method of in vitro diagnosing multiple sclerosis, an early stage or a prestage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to the invention comprising contacting a sample of body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent according to the invention and determining the presence of bound antibody from the sample.

In yet another aspect, the invention relates to a method of in vitro diagnosing multiple sclerosis or a prestage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to the invention comprising contacting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent according to the invention and determining the presence of bound antigen from the sample.

An important embodiment of the invention relates to a method for producing an antigen or epitope characteristic to the retrovirus, which comprises cultivating cells harbouring a plasmid which contains and is capable of expressing a nucleotide sequence which codes for a polypeptide which has the properties of the antigen or epitope of the invention. An alternative method for producing an antigen or epitope characteristic to the retrovirus, comprises synthesizing a peptide having an amino acid sequence derived from the nucleotide sequence according to the invention.

The term "target nucleotide sequence" describes any nucleotide sequence as defined above, preferably an oligonucleotide sequence, which contains a nucleotide sequence complementary to the probe used in the various assays such as PCR or hybridization.

In a further aspect, the invention relates to a method for obtaining a protective immunity in an animal, including a human being, against multiple sclerosis caused by the retrovirus of the culture according to the invention, comprising administering, to the animal, an immunogenically effective amount of a vaccine against a herpes group virus such as Epstein-Barr virus, thereby preventing the previously mentioned interaction between a herpes group virus such as Epstein-Barr virus and the retrovirus of the invention which interaction is assumed to result in the activation of the retrovirus.

The vaccine should be made so as to allow an optimal stimulation of the relevant parts of the immune system, i.e to present the immunogenic agent for a period of time and in a form being optimal with respect to the recognization, the uptake or any other interaction or processing necessary for the stimulation.

The term "vaccine" is to be understood to comprise any preparation containing an immunologically effective part of a herpes group virus, e.g. an antigen or epitope of the herpes group virus suited for administration to living organisms for the prevention of MS by providing an animal, such as a human, with a protective immunity against a herpes group virus such as an Epstein-Barr virus.

The invention also relates to a method for obtaining a protective immunity in an animal, including a human being, against multiple sclerosis caused by the retrovirus of the invention, comprising administering, to the animal, an immunogenically effective amount of a vaccine comprising any preparation containing an immunologically effective part of a cell culture infected with the retrovirus of the invention, or purified retrovirus or fragments thereof all of which are defined above, e.g. an antigen or epitope of the invention, or comprising an anti-idiotypic antibody of

the invention suited for administration to living organisms for the prevention of MS so as to prove the animal, such as a human, with a protective immunity against the retrovirus according to the invention.

The vaccines described above may be used seperately or the two vaccines may be used in combination.

The term "immunization" is understood to comprise the process of evoking a specific immunologic response with the expectation that this will result in humoral, and/or secretory, and/or cell-mediated immunity to a herpes group virus such as Epstein-Barr virus or to the retrovirus of the invention, i.e. immunity is to be understood to comprise the ability of the individual to resist or overcome infection or to overcome infection "more easily" compared to individuals who have not been immunized or to tolerate the infection without being clinically affected or to block transmission. Thus, in one aspect the immunization according to the present invention is a process of increasing resistance to infection with a herpes group virus such as Epstein-Barr virus, thereby preventing such a herpes group virus from activating any retrovirus according to the invention present in the same individual. An overall aspect in the preparation of the vaccines of the invention is the physiological acceptability of the components and of the total composition of the vaccine. The final formulation of the vaccine should be a mixture of substances supporting and enhancing the immune response induced by the specific immunogenic component. In another aspect, the immunization according to the present invention is a process of increasing resistance to infection with a retrovirus of the invention.

By using the above-mentioned vaccine against a herpes group virus such as a Epstein-Barr virus and vaccine against the retrovirus of the invention in combination, the effect of the immunization may be enhanced.

In a further aspect, the invention relates to a method as explained above, in which the vaccine is a live or dead Epstein-Barr virus administered to the mammal at a stage in the development of the mammal in which the disease caused by Epstein-Barr virus has a mild clinical cause and cannot induce active production of the retrovirus of the invention to result in multiple sclerosis, an early stage or a pre-stage thereof. The vaccine may be administered to a mammal, preferably a human, and the stage in which the administration is performed is the pre-puberty stage. The mammal, such as a human, to which the vaccine is administered may be mammal which has been shown to carry the retrovirus of the culture as defined above.

The vaccine may be an attenuated Epstein-Barr virus or an immunogenic antigen characteristic to Epstein-Barr virus and eliciting the formation of antibodies against Epstein-Barr virus.

The administration of the vaccine against the retrovirus of the invention to a mammal, such as a human, may be performed at any stage in the development of the mammal. Preferably, the vaccine against the retrovirus from the culture of the invention may be administered to a mammal which has been shown to carry the retrovirus of the culture as defined above or which has been shown to carry a herpes group virus such as an Epstein-Barr virus.

Legend to figures

Figure 1a.

An Epstein-Barr virus particle with a diameter of 150 nm is seen outside the cell membrane of a B-cell (EBV), i.e. a core surrounded by an icosahedral capsid measuring maximally 100nm in diameter and an outer irregularly shaped envelope with projections.

Figure 1b.

Eppstein-Barr viral capsids are seen in the nucleus (EBV.VC). The central nucleic acid of the virion is surrounded by an icosahedral capsid (Crawford and Edwards, 1990).

Figures 1c and 1d.

Retrovirus-like particles located just outside the cell membrane measuring 90-100 nm in diameter (RVLP). The particles have a core-like condensation and the envelope does not contain visible projections. Note the variable size of the particles and the diffuse spherical cores as those illustrated in a textbook on human retroviruses (Dalgleish and Weiss, 1990).

Figure 2a.

Viral particles together with a mixture of cell organelles and other small "cell-parts" are seen. The material is a pelleted fraction of a sucrose gradient centrifuged as described in Example 5. After resuspension of the pellet, the material is spread on grids and stained as described in Example 8.

Figure 2b.

Viral particles captured by monoclonal antibodies to gp46 are seen. The material is prepared as described above in Figure 2a. Small cellular fragments have been washed away as described in Example 8. Finally, the material is stained.

EXAMPLE 1

Cultivation of the lymphoblastoid cell culture

The lymphoblastoid B-cell line was established as follows. Heparinized blood was diluted in phosphate-buffered saline PBS (pH 7.4, 20 i.u./ml heparin), before separating the mononuclear cells by Ficol-Isopaque density gradient centrifugation. The mononuclear cells were harvested and washed twice in cold PBS. Finally the mononuclear cells were seeded at a density of $20x10^6$/5 ml and grown in RPMI 1640 (Seromed) supplemented with 200 i.u./ml penicillin (Leo), 0.2 mg/ml streptomycin (Rosco), 0.29 mg/ml glutamine (Sigma), 0.01 M hepes buffer (Bioproduct), 5% human heat inactivated sera in Falcon Primaria bottles. During the first 7 weeks of cultivation, the serum percentage was 5%. After appearances of clones in the culture, the medium contained 10% of human serum.

In the lag period before appearance of the clones in the cell culture, the morphology of the cells was studied. The non-adherent cells became fewer and small. The adherent cells which were initially small and round became increasingly larger with formation of multinucleated giant cells. After 6 weeks of culture, few large multinucleated giant cells with 12 to 17 nuclei were present and small clones of B-cells were attached to these cells. By the 7th week of culture, the B-cell clones were more numerous and were also seen in the culture without attachment to adherent cells. After 7 weeks of culture, the B-cell clones were subcultured in medium containing different percentages of human serum. It was found to be essential for survival of the culture that the cell density was initially not below $0.5x10^6$ cells/ml, and only half of the medium was changed when subcultured. Furthermore, it was found that medium containing 10% of human serum supported the growth of the cell line better in long-term culture than a similar medium containing fetal calf serum. The cells were subcultured

three times a week with a split ratio of 1:3 or 1:4 with a cell density of $0.5x10^6$ cells/ml.

The cells were capable of growing in clumps or singly without adherence to the surface of the container in which the cultivation took place. The population doubling time was initially 17 hours but rose to 26 hours after 5 months of culture. The maximum cell density obtainable was around $2x10^6$ cells/ml and a poor cell proliferation was observed at cell densities below $0.35x10^6$ cells/ml.

EXAMPLE 2

Examination of the lymphoblastoid cell culture using transmission electron microscopy

Materials and methods

Fixation

$2x10^6$ cells were fixed with cold 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate for at least 1 hour. The cells were then pelleted by centrifugation at 1500 rpm for 5 minutes. The pellet was washed 3 times in cacodylate buffer, pH 7.2 and then postfixed for 1 hour in 1% osmium tetroxide buffered at pH 7.2 with veronal acetate buffer and then washed 3 times in veronal acetate buffer. Between these washing steps, pelleting was carried out at 1500 rpm for 5 minutes.

Block staining

$2x10^6$ cells were washed 3 times with maleate buffer, pH 5.2, and stained for 1 hour in 0.05 M maleate buffer + 0.5% uranyl acetate, pH 6.0. They were then washed 3 times with maleate buffer, pH 5.2. Between these washing steps, pelleting was carried out at 1500 rpm for 5 minutes.

Dehydration and embedding

$2x10^6$ cells were dehydrated in increasing concentrations of alcohol (70%, 90%, 96% absolute alcohol), followed by 3 changes in propanol. Between these dehydration steps, pelleting was carried out at 1500 rpm for 5 minutes.

The cells were then treated for 1 hour in 75% propanol + 25% TAAB812 resin mixture (TAAB Laboratories), for 1 hour in 50% propanol + 50% TAAB812 resin mixture, and for 1 hour in 25% propanol + 75% TAAB812 resin mixture, followed by 24 hours in fresh resin mixture.

Between each of these treatments, pelleting was carried out at 2000 rpm for 10-15 minutes.

After blocking, the resin was polymerized for 48 hours at 60°C.

Sectioning and staining

Sections for electron microscopy of light grey or grey in colour (about 40 nm) were cut on an LKB Ultratome with a diamond knife. They were then stained with uranyl acetate for 10 minutes, followed by lead citrate for 2 minutes.

Electron microscopy

The sections were examined in a Jeol 100 B electron microscope at an accelerating voltage of 60 KV.

Particles with herpes group virus morphology were observed both inside and outside some of the cells as shown in Figure 1a. Immature vial capsids were seen in the nucleus (Figure 1b). Viral capsids without core were also seen. Another type of particles, that is, the retrovirus according to the invention, was seen along the outer cell mem-

brane in approximately 1-2% of the cells. The particles were spherical structures with a diameter of 90-100 nm, containing a core-like condensation as seen in Figures 1c and 1d. The outer membrane did not contain visible projections. The structures were indistinguishable from virus particles and had a close resemblance to known retrovirus. Due to the morphology of these particles, the retrovirus was characterized as being a type C-like retrovirus in accordance with the description given in Dalgleish et al., 1990.

EXAMPLE 3

Characterization of the lymphoblastoid cell culture

The cell culture was examined for production of various products, and the presence of leukocyte differentiation antigens was analyzed.

The presence of interleukin-6 (IL-6) was measured by a biological assay using the IL-6 sensitive B_9 cell-line as described in Rozenberg et al., 1991. Interferon assay was performed as described in Haahr et al., 1976, and neutralization was made with a polyclonal antibody to interferon-β-(Boehringer Mannheim).

Tumour necrosis factor activity was measured in a cytotoxicity assay in L-929 cells. In brief, monolayers of cells seeded the day before ($2x10^4$ cells/well) were overlayed with two-fold dilutions of the supernatants to be tested, obtained from the supernatant of cell culture of the cell line 1533, and incubated at 38.5°C with actinomycin D (1 μl/ml; Calbiochem, Behring Diagnostics, La Jolla) for 18 hours. The plates were fixed in 1% formaldehyde and stained with crystal violet (1 mg/ml) for 20 minutes, washed and read for light absorbance at 600 nm in an ImmunoReader (InterMed NJ-2000). TNF titers were assessed as the dilu-

tion resulting in 50% cytotoxicity and compared with standard TNF titrated on each plate. The cytotoxic substance was identified as TNF-β by neutralization by the specific antiserum to human TNF-β (rabbit anti-human TNF-β polyclonal antibody 80 µl/ml (80-800 neutralizing units), Genzyme) and was not neutralized by specific antiserum to human TNF-α (rabbit anti-human TNF-α 80 neutralizing units/ml, Walther Fies Gent).

The cell culture was shown to have an autocrine production of interleukin-6 (2 units/ml) and to produce interferon-β spontaneously (2.74 units/ml). Tumour necrosis factor-β (100 units/ml) was also produced by the cell culture spontaneously.

The identification of the presence of leukocyte differentiation antigens was performed by the use of the immunohistochemical method as described in the following.

Cytocentrifuge specimens were air-dried and fixed in acetone for 10 minutes at room temperature whereafter the specimens were stained with each of the monoclonal antibodies listed in Table 1 below using the alkaline phosphatase anti-alkaline phosphatase (APAAP) or a three-stage immunoperoxidase method as described in Pallesen et al., 1991. Phenotypic characterization of the lymphoblastoid cells was performed with a wide panel of monoclonal antibodies to leukocyte differentiation antigens in accordance with principles described in Pallesen, 1988.

The results obtained appear from Table 1 below. The cell population did not contain cells labelled with T-lymphocyte- or macrophage-specific monoclonal antibodies.

TABLE 1

Expression of leukocyte differentiation antigens of the LCL MS 1533

	Reactivity[b]
B-cell antigens[a]	
CD19 (DAKO-CD19)	2+
CD20 (L26)	2+
CD21 (OKB7)	3+
CD22 (To15)	1+
CD23 (MHM6)	3+
CD77 (424/4A11)	-/1+
Polytypic immunoglobulin	3+
T-cell antigens (various)	-
CD4 (DAKO-T4)	-
Monocyte and granulocyte antigens (various)	-
CD15 (DAKO-M1)	-
Cell adhesion molecules	
CD54 (LB-2)	2+
CD58 (TS2/9)	3+
CD11a (F110.22)	2+
Activation-associated antigens	
CD30 (Ber-H2)	3+
CD25 (Tü69)	-/1+
HLA antigens	
HLA-I (DAKO-HLA-ABC)	3+
HLA-II (Tü35)	3+
Proliferation-associated antigens (Ki-67)	95%

a) Monoclonal antibody designation is indicated in brackets.
b) Score: - = negative, + = positive, -/+ = <50% cells positive, 1+, 2+, 3+ = weak, medium and strong staining, respectively.

EXAMPLE 4

Testing for various retrovirus-related antigens in the cell culture infected with the retrovirus

The lymphoblastoid cell culture was examined by an immunofluorescence analysis for the expression of the HTLV-I antigens p19 and p24 by the use of the following monoclonal antibodies directed against HTLV-I p19 and p24: 12G4, MAS 197b, 6G9 and MAS 199b (Sera-Lab).

As appears from Table 2 in Example 9 below, no binding of monoclonal antibodies directed against the HTLV-I antigens p19 and p24 was observed, which means that the lymphoblastoid cell culture did not express these antigens.

To characterize other possible antigens present in the lymphoblastoid cell culture infected with the retrovirus, the culture was examined for animal virus antigens from murine-leukemia virus (MuLV), simian sarcoma virus-1 (SSV-1, p28), feline leukemia virus (FeLV) and the endogenous cat virus (RD114) using immunofluorescence analysis. The polyclonal antibodies used were provided by Björn Nexö, The Fibiger Laboratory, Copenhagen, Denmark (see Kaltoft et al., 1988).

As appears from Table 2, no binding of antibodies was observed, which means that the lymphoblastoid cell culture infected with the retrovirus did not express antigens characteristic of the above-mentioned animal viruses.

Finally, the antibodies present in the serum from the patient from which the lymphoblastoid cell culture was derived were examined in order to further support the above results showing that the retrovirus from the lymphoblastoid cell culture was not an HIV-I, HIV-II or HTLV-I virus. By use of ELISA, the serum from the patient was examined for IgG antibodies to HIV-I and HIV-II, and HTLV antibodies were determined by an immunofluorescence method at the State Serum Institute, Copenhagen. No positive result was obtained showing that no antibodies capable of binding to HIV-I, HIV-II or HTLV-I viruses were produced as a result of infection with the retrovirus from the lymphoblastoid culture. Furthermore, as there is no serological difference between HTLV-I and HTLV-II with respect to the antibodies produced in an infection, the retrovirus from the cell culture was not an HTLV-II virus.

EXAMPLE 5

Purification of viral particles or antigens from the lymphoblastoid cell culture

Several approaches were used in the purification of viral particles or antigens. The principles of this type of purification are outlined in Poiesz et al., 1980.

A. Sucrose gradients

$1x10^8$ cells were sonicated and harvested for 10 minutes by centrifugation at 2000 rpm in a Sorvall GLC-2 centrifuge, and the pellet of the supernatant was layered on the following gradient after the supernatant had been spun for 3 hours at 19,000 rpm in a VTi 55 rotor (30,000 x g) at 4°C, and the pellet had been resuspended in 1-2 ml of TNE (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA):

25% sucrose: 12.5 g of sucrose in 25 ml of 2xTNE (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM EDTA) and 25 ml of distilled water

45% sucrose: 22.5 g of sucrose in 25 ml of 2xTNE (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM EDTA) and 25 ml of distilled water

The gradient mixer was rinsed and the gradient was made up in 9/16x3 1/2 (14 x 89 mm) tubes.

The gradient was spun for 16 hours at 38,000 rpm in an SW41 rotor at 4°C, and 6 fractions were taken and diluted three times with TNE (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA). Centrifugation was then performed for 3 hours at 38,000 rpm in an SW41 rotor (45,000 x g) at 4°C, and the retrovirus fraction was then contained in the pellet.

No bands were visible in the first gradients which were divided into 6 fractions. Each fraction was pelleted and 6 new gradients were run separately.

B. Purification by adsorption to antibody-conjugated micro-beads

The labelling of microspheres with rabbit anti-mouse/anti-rat IgG was performed as follows:

1 g of beads (Tessek, Aarhus; = 3 x 10^9 beads/g) was washed in 10 ml of Buffer A (700 mM $(NH_4)_2SO_4$; 100 mM sodium borate, pH 9). The beads were spun down and the supernatant was removed. 2-5 ml of IgG (5-10 mg/ml IgG) in Buffer A was added, and the mixture was shaken overnight at room temperature. The beads were again spun down and the supernatant was removed. The mixture was washed several times in double distilled water and OD_{280} was read in a photometer (approximately 0 if washed efficiently). The mixture was then suspended in 1 ml of Buffer B (100 mM sodium borate; 100 mM ethanol amine, pH 9) and incubated overnight at room temperature. The beads were spun down and washed in double distilled water until pH was about 6-7. The mixture was washed and resuspended in PBS.

The antibodies were conjugated to the labelled microspheres (about 3x10^9 spheres/g were used) in the following manner: incubation was carried out with 1 ml of antibodies: anti-rat HTLV-I: 30g, 1e, 5a, 69b; or anti-mouse HTLV-I 46 (T. Schultz, Chester Beatty Lab., UK) (+), or the following antibodies raised against other HTLV-I antigens: anti-mouse HTLV-I p19 (ascites) or anti-mouse HTLV-I p24 (K. Kaltoft, Bartholin, Aarhus University) (-) 1:3000 x diluted in PBS for 1 hour at room temperature. The beads were spun down and washed several times in PBS/Tween.

The incubation with sample was performed by adding 10^4

spheres to 500 µl of sample supernatant. The mixture was then incubated at 37°C while shaking.

Two methods were employed to remove bound particles and IgG. Either the beads were incubated for 5 minutes at room temperature with 100 mM glycine, pH 2.5, followed by neutralization by addition of 1/10 volume of sample of 1 M Tris-HCl, pH 9. Alternatively, the beads were incubated with 4 M $MgCl_2$ for 5 minutes at room temperature, followed by 100x dilution with PBS, pH 7.4.

C. Purification of membrane-associated proteins by Triton X-114 temperature induced phase separation

5 x 10^8 cells were harvested and washed twice in PBS, pH 7.4 containing 1 mg/ml BSA.

The cells were resuspended and lysed in 4 ml of 100 mM Tris-HCl, 1% Triton X-114, 10 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), pH 8.1 at 0°C for 10 minutes.

The lysate was clarified by centrifugation at 5000 x g for 10 minutes at 4°C and was transferred to Falcon tubes and incubated for 10 minutes at 37°C to induce the phase separation.

The detergent phase at the bottom of the tube was collected after centrifugation for 10 minutes at 20°C and 1800 x g. An equal volume of 100 mM Tris-HCl, pH 8, was added and the procedure was repeated.

The detergent phase (100-600 µl) was suspended in 2 ml of 100 mM Tris-HCl, pH 8, with 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate; Boehringer) to avoid temperature-induced phase separation during subsequent handling.

For Western blots, an equal volume of sample and sample buffer was used, but the final SDS concentration should not exceed 0.1%.

The viral particles or antigens prepared by these methods were used to further characterize the virus as exemplified in the following.

EXAMPLE 6

Reverse transcriptase assays

The supernatants obtained from cell line 1533 were concentrated and purified by ultracentrifugation on sucrose gradients, followed by pelleting of the various fractions as described in Example 5 above.

20 µl of the test material thus obtained was placed in a Widal glass (3 ml) and to this the following mixture was added:

5 µl of Tris 0.5 M/DTT (dithiothreitol) 0.04 M (pH 8.2)
5 µl of NaCl 0.1 M
5 µl of $MgCl_2$ 0.3 M
23 µl of double distilled water
10 µl of NP-40 2% (Boehringer)
2 µl of poly rC - oligo dG_{12-18} (Pharmacia Prod.No. 27-7944)
10 µl of Deoxy{8-^{3}H} guanosine-triphosphate (Amersham TRK.350)

The test tubes were incubated at 37°C for 90 minutes. The reaction was stopped by adding 100 µl of a cold solution of distilled water containing 12.5% of water saturated with sodium phosphate, 12.5% of water saturated with sodium pyrophosphate, and 20% of trichloroacetic acid (TCA). After 30 minutes on ice, the tubes were emptied onto a Millipore

membrane filter (cat.no. GVWP02500), and the membrane filter was washed 4 times with 5 ml of 5% TCA under depression of 2 atm. The membrane filters were dried, placed in counting tubes, covered with 5 ml of scintillation fluid (Lumasafe-Packard), and counted in a beta-counter (Packard) for 5 minutes per test tube. Results were measured in counts per minute (CPM). The various samples were run in duplicate. The principles of this assay are described in Poiesz et al., 1980.

Results

During the initial phases of the growth of MS 1533, the supernatants were tested either unconcentrated or concentrated to a level of concentration lower than what can be obtained by centrifugation on sucrose gradients by a method essentially as outlined above. Tests run on the former material only showed borderline reverse transcriptase activity. In the purified material, a reverse transcriptase activity 3 fold background was found.

EXAMPLE 7

Testing purified retrovirus for various retrovirus-related antigens

Retroviral particles or antigens purified by methods A, B and C as described in Example 5 above were subjected to SDS-PAGE. The 10% SDS-PA gels were subsequently used either for direct visualization of the proteins in the samples by silver staining or for Western Blotting. In either case, 5 μl of prestained M_r marker (Sigma) was loaded in one lane of the gel.

Silver staining

Gels to be silver stained were run on the Pharmacia PHAST System. The samples were prepared before loading by mixing 10 µl of sample with 2.5 µl of sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 12% SDS and 5% BPB) and 1.25 µl of 100 mM DTT. The samples were boiled for 3 minutes, and 1.4 µl of iodoacetamide was added. The gels were run at 50 V overnight.

After electrophoresis, the gels were silver stained by incubating the gels consecutively in the following:

1 hour at room temperature in 150 ml of 96% ethanol; 20 ml of 100% HAc; 120 ml of distilled water,

1 hour at room temperature in 15 ml of 96% ethanol; 21 ml of 100% HAc; 264 ml of distilled water,

1 hour at room temperature in 120 ml of glutardialdehyde; 180 ml of distilled water; washing in 300 ml of distilled water 3 x 10 minutes, 1 x 1 hour, 1 x 10 seconds,

30 minutes at room temperature in 97,5 µl of 100 mM DTT; 300 ml of distilled water; washing in 300 ml of distilled water 2 x 5 minutes,

30 minutes at room temperature in 300 ml of distilled water with 0,3 g $AgNO_3$, 1 minute, followed by 3 minutes in 150 µl of 37% formalin in 300 ml of 3% Na_2CO_3,

10 minutes at room temperature in 200 ml of distilled water with 4,85 g of citric acid, washing in 300 ml of distilled water 2 x 30 minutes; washing in 50 ml of 96% ethanol; 6,8 ml of 87% glycerol; 143 ml of distilled water,

and drying the gel on a paper towel.

Western Blotting

The samples were prepared before loading by mixing 30 µl of sample with 7.5 µl of sample buffer (100 mM Tris-HCl pH 6.8, 10% glycerol, 12% SDS and 5% BPB) and 3.7 µl of 100 mM DTT. The samples were boiled for 3 minutes, and 4.4 µl of iodoacetamide was added. The gels were run at 50 V overnight.

After electrophoresis, the gels were blotted to Immobilon (Millipore) membrans in a Kem-En-Tec wet blotter. The gels were equilibrated in transfer buffer for 15 minutes, while the membranes were equilibrated in methanol, water and transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 20% ethanol; 0.1% SDS).

The gels and membranes were made up as sandwiches with Whatman filter paper and blotted 500 Vh (overnight at 25 V). The sandwich was dismantled and the membrane washed in double distilled water. The membrane was then blocked in TBS/0.1% Tween for 15 minutes at room temperature and could be stored as such in TBS/Tween at 4°C for a week.

The membranes were screened with the following antibodies, all raised against HTLV-I envelope antigens: anti-rat HTLV-I: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I 46 (T. Schultz, Chester Beatty Lab., UK) (+), and the following antibodies raised against other HTLV-I antigens: anti-mouse HTLV-I p19 (ascites) and anti-mouse HTLV-I p24 (K. Kaltoft, Bartholin, Aarhus University) (-).

The screening procedure was as follows: Antibodies were 1:3000 diluted in PBS with 0.05% Tween, incubated overnight at room temperature on a shaker and washed 3 x 5 minutes in TBS with 0.05% Tween and 350 mM NaCl. The membranes were then incubated for 1 hour with 1:3000 diluted rabbit anti-mouse or anti-rat antibodies and washed 3 x 5 minutes in TBS with 0.05% Tween and 350 mM NaCl. The membranes were

then incubated for 1 hour with 1:5000 diluted AP-conjugated goat anti-rabbit antibodies and washed 3 x 5 minutes in TBS with 0.05% Tween and 350 mM NaCl. The membranes were then washed for 5 minutes at room temperature in 100 mM ethanol amine, pH 9, and the substrate (1/10 vol NBT (4-nitroblue tetrazolium chloride; Boehringer); 1/100 vol of BCIP (5-bromo-4-chloro-3-indolyl phosphate; Boehringer); 1/500 vol of 2 M $MgCl_2$) in 100 mM ethanol amine, pH 9, was added. The membranes were washed in water after the colour reaction.

The fact that some, but not all, of the antibodies gave a positive reaction (+) indicates similarity to, but not identity with HTLV-I.

EXAMPLE 8

Electron microscopy - negative staining

A small drop of virus containing solution from the double purified fractions as described in Example 5A was placed on carbon coated G400 grids (Gilder) which were initially coated with parlodion 2% in amyl acetate, and a drop of negative stain (1% phosphotungstic acid (PTA) adjusted to pH 6.5 with 1N KOH) was added. After 20 seconds, excess moisture was removed with the torn edge of a filter paper. The grids were then air-dried and examined in an electron microscope (Jeol 100 B, Jeol, Japan). The result of one of the above-described experiments is illustrated in figure 2a.

Immunoelectron microscopy

The grids were floated (carbon side down) on a 15 μl drop of a solution of protein A (0.01 mg/ml in PBS buffer) for 10 minutes, and excess moisture was removed with the torn edge of a filter paper. The grids were washed by transferring them through 3 drops of PBS buffer, 1-2 minutes on top

of each drop. The grids were floated on 15 µl of antiserum (mouse monoclonal antibody to HTLV-I gp46 diluted 1:100 in PBS) for 10 minutes. Excess moisture was removed with the torn edge of a filter paper, and the grids were then washed in buffer as described. The grids were then floated on 15 µl of virus-containing solution from the double purified fractions as described in Example 5A and were washed in buffer as described. Then a drop of negative stain (potassium phosphotungstate (PTA)) was placed on the grids, and excess moisture was removed with the torn edge of a filter paper. Excess stain was removed after 20 seconds, and the grids were air-dried and examined in an electron microscope (Jeol 100 B, Jeol, Japan). The result of one of the above-described experiments is illustrated in figure 2b.

EXAMPLE 9

Testing the culture containing the retrovirus for nucleotide sequences from HIV-I and HTLV-I

Various PCR methods were employed to identify and partly characterize the nucleic acids of retroviral origin in MS 1533. PCR strategies were performed as described in Innis et al., 1990 (PCR Protocols, A guide to methods and applications, Academic Press, Inc.). In order to avoid endogenous retroviral sequences, cytoplasmatic RNA was purified, subjected to reverse transcription and subjected to PCR analysis. 1st strand cDNA was used as a template in all PCR experiments described herein. Cytoplasmatic RNA was purified and amplified as follows:

Approximately 120 x 10^6 cells were harvested for 5 minutes at 1000 rpm in a centrifuge and were then rinsed twice in PBS. The cells were resuspended in 800 µl of PBS in an Eppendorf tube and harvested for 10 seconds in an Eppendorf centrifuge after which the supernatant was discarded. The cells were lysed in 500 µl of lysis buffer (140 mM NaCl,

1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.6, 0.5% NP-40) and were placed on ice for 5 minutes. The mixture was pelleted cold for 5 minutes at 10,000 x g in the Eppendorf centrifuge.

To the supernatant was added sodium acetate, pH 7.0, to 150 mM and SDS to 1%, after which 5 µl of 20 mg/ml proteinase K (Merck) was added and the suspension was incubated at 37°C for 30 minutes. Phenol/chloroform extraction was performed twice; chloroform extraction was performed once. The aqueous phase was adjusted to 300 mM sodium acetate, pH 7, and 2.5 volumes of 96% ethanol was added. The RNA was harvested cold for 30 minutes, washed with 80% ethanol and resuspended in distilled water.

Detection of radioactively labelled PCR products was accomplished by end labelling the oligonucleotide primers as follows:

The following mixture was prepared:

1 µl 10 pmol/µl oligonucleotide (prepared synthetically), 2 µl of 10 x T_4PNK buffer (commercially available buffer from Boehringer Mannheim), 5 µl of γ^{32}P-ATP (from NEN, New England Nuclear), 11.4 µl of distilled water, and 8 units (1 µl) of T4-polynucleotide kinase.

The mixture was incubated for 45 minutes at 37°C and for 10 minutes at 68°C. To the mixture was added 40 µl of distilled water, 240 µl of 7 M NH_4Ac and 750 µl of 96% ethanol, and the tube was placed on an ice bath for 30 minutes.

The mixture was harvested for 20 minutes at 12,000 rpm (15,000 x g) in an Eppendorf centrifuge at 4°C and then washed with 80% ethanol. The mixture was resuspended in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8).

First strand cDNA synthesis and PCR were performed with equipment and kits from Perkin Elmer Cetus (USA). Reverse

transcription was performed in a buffer with 5 mM $MgCl_2$, 1x PCR, 1 mM of each of the dNTP's, 1 μ/μl RNase inhibitor, 2,5 μ/μl reverse transcriptase, 2.5 μM random hexamers and 1 μg of RNA. The mix was incubated at 42°C for 15 minutes, at 99°C for 5 minutes, and at 5°C for 5 minutes. Amplification was performed by adding $MgCl_2$ to 2 mM, 1x PCR, 2.5 μ Taq DNA polymerase and double distilled water to 100 μl. In low stringency reactions, the annealing temperature was between 37°C and 48°C, in high stringency reactions it was 60°C. These criteria generally apply, where "low stringency PCR conditions" and "high stringency PCT conditions", respectively, are referred to in the present specification and claims.

The lymphoblastoid cell culture infected with the retrovirus was analyzed for the presence of nucleotide sequences specific to the retrovirus HIV-I using high stringency nested PCR as described in Teglbjærg et al., 1992. The following primer pairs and probes were used:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK30, SK70, SK19 and SK31.

The DNA sequences of the primers and probes were as follows:

Primers:

LST1: 5' ATC AAG CAG CCA TGC AAA TG
LST2: 5' CTA CAT AGT CTC TAA AGG GT
SK38: 5' ATA ATC CAC CTA TCC CAG TAG GAG AAA T
SK39: 5' TTT GGT CCT TGT CTT ATG TCC AGA ATG C
LST3: 5' AGG AGG AGA TAT GAG GGA CAA TTG
LST4: 5' GGA GCT GTT GAT CCT TTA GGT ATC
SK68: 5' AGC AGC AGG AAG CAC TAT GG
SK69: 5' CCA GAC TGT GAG TTG CAA CAG
LST5: 5' GCC TGG GAG CTC TCT GGC TA
LST6: 5' CGG GCG CCA CTG CTA GAG A
SK29: 5' ACT AGG GAA CCC ACT GCT

SK30: 5′ GGT CTG AGG GAT CTC TA

Probes:

SK19: 5′ ATC CTG GGA TTA AAT AAA ATA GTA AGA ATG TAT AGC CCT AC

SK70: 5′ ACG GTA CAG GCC AGA CAA TTA TTG TCT GGT ATA GT

SK31: 5′ ACC AGA GTC ACA CAA CAC ACG GGC ACA CAC TAC T

The lymphoblastoid cell culture was also analyzed for the presence of nucleotide sequences specific to the retrovirus HTLV-I using PCR. The following primers were used:

HTLV-I/026 and HTLV-I/029

HTLV-I/026: 5′ GAG GCA GAT GAC AAT GAC CAY GAR CC

HTLV-I/029: 5′ NAG CCA CCT NCT GAA CTG TC

As appears from Table 2 below, no nucleotide sequences specific for HIV-I or HTLV-I were detected using high stringency PCR conditions.

Low stringency annealing

Using low stringency PCR conditions and a panel of primer pairs derived either from HTLV-I or from known retroviral consensus sequences, PCR products were obtained (indicated as positive results in Table 2). The results of the PCR experiments are illustrated in Table 2. The primer sequences were based on known sequences from other retroviruses. The sequences were based on various principles for reverse translation. The following primers were synthesized and used:

477/478, 1898/1899, 1900/1901, 1956/1957 and 2345/2346. Their sequences were as follows:

477: 5′ AAT TGA AGC GAG CTG ATT GGT TAG TTT

478: 5′ AAT TAA ACT AAC CAA TCA GCT CGC TTC

1898: 5′ CTC GTC GAC AAC ATG TCA TCA ATG TA

1899: 5′ CTC GAA TTC GAT GCA TAT TTC ACA ATA CC

1900: 5′ CTC GAA TTC TAA AAG ATG CCT TCT T

1901: 5′ CTC GTC GAC ATC GTC CAC ATA GGA

1956: 5′ GAG GCA GAT GAC AAT GAC CAY GAR CC

1957: 5′ NAG CCA CCT NCT GAA CTG TC

2345: 5′ TGG AAT GTI CTI CCI CAI GG

2346: 5′ GCT AGG AGA ATI TCI TCC ATI TA

The following oligonucleotides for PCR are contemplated to be useful in the further analysis of the nucleotide sequence of the retrovirus:

1. 5′ TGC AAG GCC CTG CAG GAG CAG TGC TGC

2. 5′ TGG GGC CTG AAC TGG GAC CTG GGC CTG TCC CAG TGG

3. 5′ CCA CTG GGA CAG GCC CAG GTC CCA GTT CAG GCC CCA

4. 5′ CGG TAC CCC CAC TAC TCC

5. 5′ GGA GTA GTG GGG GTA CCG

6. 5′ AAC ACA GAG CCC TCC CAG CTG CCC

7. 5′ CCT GTG CCC ACC CTG GGC TCC CGG T

8. 5′ ACC GGG AGC CCA GGG TGG GCA CAG G

9. 5′ ATC CCC TGG AAG TCC AAG CTG CTG ACC

10. 5′ CTG GTG CAG CTG ACC CTG CAG TCC

11. 5′ AAC TGG ACC CAC TGC TTT GAC CCC CAG ATC CAG

12. 5′ CAC TGG CAT GTG CTG TAC TCC CCA AT

13. 5′ ATT GGG GAG TAC AGC ACA TGC CAG TG

TABLE 2

Antibody, PCR and Immunofluorescence analysis

Antibody against the following retroviruses:	
HIV-I	negative
HIV-II	negative
HTLV-I	some negative, some positive
High stringency PCR analysis for the following retroviruses:	
HIV-I	negative
HTLV-I	negative
Low stringency PCR analysis for the following retroviruses:	
HTLV-I	positive
various retroviral consensus	positive
Immunofluorescence analysis for HTLV-I antigens:	
p19	negative
p24	negative
Immunofluorescence analysis for animal virus antigens:	
MuLV	negative
SSV-1, p28	negative
FeLV	negative
RD114	negative

EXAMPLE 10

Analysis of the lymphoblastoid cell culture for Epstein-Barr virus encoded proteins.

Immunohistochemistry was performed as described in Pallesen et al., 1991 in order to analyze the lymphoblastoid cell culture for expression of Epstein-Barr virus proteins. The expression of the latent infection protein EBNA 1 was determined by a complement fixation assay as described in Reedman & Klein, 1973. The expression of the latent infection proteins EBNA 2 and LMP was determined using the monoclonal antibodies PE2 and CS.1-4 which were obtained from L.S. Young and M. Rowe (Birmingham, UK). To determine the expression of immediate-early proteins BZLF1, the monoclonal antibody BZ1 also obtained from L.S. Young and M. Rowe (Birmingham, UK) was used. Finally, the determination of the expression of productive cycle proteins, EA, VCA and MA, was performed by the use of the monoclonal antibodies 37G11, 2F2 and 11D7 obtained from Prof. H. Wolf (Munich, Germany). A detailed characterization of these antibodies has recently been published (Pallesen et al., 1991; Pallesen et al., 1991).

TABLE 3

Expression of Epstein-Barr virus encoded proteins in the LCL MS 1533

	Antigen[a)]	% positive[b)]
Latent infection proteins:	EBNA 1	100%
	EBNA 2 (PE2)	100%
	LMP-1 (CS.1-4)	91%
Immediate-early protein:	BZLF1 (BZ1)	9%
Productive cycle proteins:	EA-D (37G11)	3%
	VCA (2F2)	0.1%
	MA (11D7)	0.1%

a) Monoclonal antibody indicated in brackets.
b) Percentage of cells labelled.

The results obtained appear from Table 3 above and from the following. The latent infection protein EBNA 2 was localised to the nuclei, but occasionally, cells showed additional fine granular cytoplasmic labelling. The latent infection protein LMP was localised to the cytoplasm and cell membrane. The immediate-early proteins BZLF1 were localised to the nucleus but a weaker, diffuse cytoplasmic staining was also seen in many cells, either alone or in combination with nuclear labelling. Productive cycle proteins in the form of EA were seen as an intense nuclear and a weaker cytoplasmic reaction, whereas VCA was mainly nuclear and with a granular appearance. MA was localised to the cytoplasm.

The above results showed that the lymphoblastoid cell culture expressed latent infection and partly productive cycle proteins, indicating that the cell line was transformed by Epstein-Barr virus with an increased frequency of cells entering the lytic cycle. During the first month of cultivation, 1 to 5% of the cells were positive for VCA, but after 5 months this percentage was reduced to 0.1%.

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 16, line 19 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☒ [3]

Name of depositary institution [4]

European Collection of Animal Cell Cultures

Address of depositary institution (including postal code and country) [5]

Porton Down, Salisbury, England (WILTS SP4 0JG)

Date of deposit [6]	Accession Number [6]
4 October, 1991	V91100401

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 16, line 28 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

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Address of depositary institution (including postal code and country) [4]

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Date of deposit [5]	Accession Number [6]
8 April, 1992	W 92040805

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

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E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Ulla P. Gertsen

(Authorized Officer)

Ulla Gertsen
Head Clerk

☒ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Claims

1. A cell culture comprising cells which are infected with a type C-like retrovirus which is present in human patients who have symptoms indicating an early stage of multiple sclerosis, the retrovirus being a retrovirus which can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the cell culture using the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK30, SK70, SK19 and SK31

and in PCR using the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the cell culture,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the cell culture.

2. A cell culture comprising cells which are infected with a type C-like human retrovirus, the retrovirus being a retrovirus which can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

a) in which the retrovirus shows the following negative tests:

A) in nested PCR analysis on the cell culture using high stringency PCR conditions, the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK30, SK70, SK19 and SK31

and in PCR analysis using high stringency PCR conditions, the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the cell culture,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the cell culture,

and the retrovirus showing the following positive tests:

A) in Western blotting performed on retrovirus-containing material purified by sucrose gradient purification or Triton X-114 temperature induced phase separation or purified by adsorption to antibody-conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable,

B) in reverse transcriptase assay performed on retrovirus-containing material doubble purified on sucrose gradients, revers transcriptase activity is detectable,

c) in PCR analysis using low stringency PCR conditions and using the following primer pairs

477/478, 1898/1899, 1900/1901, 1956/1957 and 2345/2346,

PCR products are detectable.

3. A cell culture according to claim 1 or 2, in which the spherical particles have substantially the appearance as shown in Fig. 1c and 1d in transmission electron microscopy.

4. A cell culture according to claim 3, in which the spherical particles have substantially the appearance as shown in figures 2a and 2b using negative staining electron microscopy.

5. A cell culture according to any of claims 1-4, which is capable of producing the retrovirus.

6. A cell culture according to claim 5 which additionally is infected with a herpes group virus which enhances the production of the retrovirus.

7. A cell culture comprising cells which are infected with a type C-like retrovirus which can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the cell culture using the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK39, SK70, SK19 and SK31

and in PCR using the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the cell culture,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the cell culture,

the retrovirus being the retrovirus with which cells of the cell culture MS1533 - provisional deposit no. V 91082701 are infected or a retrovirus which is identical therewith except for genetic variations which are commonly found in retroviruses and which do not change the above-defined properties of the retrovirus.

8. A cell culture comprising cells which are infected with a type C-like human retrovirus, the retrovirus being a retrovirus which can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the cell culture using high stringency PCR conditions, the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK30, SK70, SK19 and SK31

and in PCR analysis using high stringency conditions, the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the cell culture,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the cell culture,

and the retrovirus showing the following positive tests:

A) in Western blotting performed on retrovirus-containing material purified by sucrose gradient purification or Triton X-114 temperature induced phase separation or purified by adsorption to antibody-

conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable,

B) in reverse transcriptase assay performed on retrovirus-containing material doubble purified on sucrose gradients, revers transcriptase activity is detectable,

c) in PCR analysis using low stringency PCR conditions and using the following primer pairs

477/478, 1898/1899, 1900/1901, 1956/1957 and 2345/2346,

PCR products are detectable,

the retrovirus being the retrovirus with which cells of the cell culture MS1533 - provisional deposit no. V 91082701 and V 92040805 are infected or a retrovirus which is identical therewith except for genetic variations which are commonly found in retroviruses and which do not change the above-defined properties of the retrovirus.

9. A cell culture according to any of claims 7 or 8, which contains genomic fragments which can also be found by PCR in blood samples from diagnosed multiple sclerosis patients.

10. A cell culture according to any of claims 7-9, which contains antigens capable of binding antibodies which are present in sera from diagnosed multiple sclerosis patients and which are not present in sera from healthy persons.

11. A cell culture according to any of claims 1-10, which is infected with a retrovirus identical to the retrovirus

with which cells of the cell culture MS1533 - provisional deposit no. V 91100401 are infected.

12. A cell culture according to any of claims 1-11, which is infected with a retrovirus identical to the retrovirus with which cells of the cell culture MS1533 - provisional deposit no. V 92040805 are infected.

13. An purified retrovirus in the form of whole retrovirus or fragments thereof which is a type C-like retrovirus which is present in human patients who have symptoms indicating an early stage of multiple sclerosis, the retrovirus being a retrovirus which, in a cell culture, can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the retrovirus or the fragments using the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK39, SK70, SK19 and SK31

and in PCR using the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the retrovirus or the fragments,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the retrovirus or the fragments.

14. An purified retrovirus in the form of whole retrovirus or fragments thereof which is a type C-like human retrovirus, the retrovirus being a retrovirus which, in a cell culture, can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the retrovirus or the fragments using high stringency PCR conditions, the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK39, SK70, SK19 and SK31

and in PCR analysis using high stringency PCR conditions, the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the retrovirus or the fragments,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the retrovirus or the fragments,

and the retrovirus showing the following positive tests:

A) in Western blotting performed on retrovirus-containing material purified by sucrose gradient purification or Triton X-114 temperature induced phase separation or purified by adsorption to antibody-conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable,

B) in reverse transcriptase assay performed on retrovirus-containing material doubble purified on sucrose gradients, revers transcriptase activity is detectable,

c) in PCR analysis using low stringency PCR conditions and using the following primer pairs

477/478, 1898/1899, 1900/1901, 1956/1957 and 2345/2346,

PCR products are detectable.

15. A purified retrovirus in the form of whole virus or frament thereof, the retrovirus being a type C-like retrovirus which, in a cell culture, can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the retrovirus or the fragments using the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK39, SK70, SK19 and SK31

and in PCR using the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the retrovirus or the fragments,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the retrovirus or the fragments.

the retrovirus being the retrovirus with which cells of the cell culture MS1533 - provisional no. V 91082701 are infected or a retrovirus which is identical therewith except for genetic variations which are commonly found in retroviruses and which do not change the above-defined properties of the retrovirus.

16. A purified retrovirus in the form of whole virus or frements thereof, the retrovirus being a human type C-like retrovirus which, in a cell culture, can exist in the form of a spherical particle structure with a diameter of 80-120 nm containing a core-like condensation and without visible projections on its outer membrane when studied in transmission electron microscopy at a magnification of 50,000 times, the retrovirus showing the following negative tests:

A) in nested PCR analysis on the retrovirus or the fragments using the following primer pairs and probes used for HIV-I detection:

LST1/LST2, SK38/SK39, LST3/LST4, SK68/SK69, LST5/LST6, SK29/SK39, SK70, SK19 and SK31

and in PCR using the following primers used for HTLV-I detection:

HTLV-I/026 and HTLV-I/029

no genomic sequences are detectable,

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the retrovirus or the fragments,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the retrovirus or the fragments

and the retrovirus showing the following positive tests:

A) in Western blotting performed on retrovirus-containing material purified by sucrose gradient purification or Triton X-114 temperature induced phase separation or purified by adsorption to antibody-conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable,

B) in reverse transcriptase assay performed on retrovirus-containing material doubble purified on sucrose gradients, revers transcriptase activity is detectable,

c) in PCR analysis using low stringency PCR conditions and using the following primer pairs

477/478, 1898/1899, 1900/1901, 1956/1957 and 2345/2346,

PCR products are detectable,

the retrovirus being the retrovirus with which cells of the cell culture MS1533 - provisional no. V 91082701 and provisional no. V 92040805 are infected or a retrovirus which is identical therewith except for genetic variations which are commonly found in retroviruses and which do not change the above-defined properties of the retrovirus.

17. A retrovirus according to any of claims 15 or 16, which contains genomic fragments which can also be found by PCR in blood samples from diagnosed multiple sclerosis patients.

18. A retrovirus according to any of claims 15-17, which contains antigens capable of binding antibodies which are present in sera from diagnosed multiple sclerosis patients.

19. An antigen or epitope derived from, produced by, or induced by the retrovirus with which the cell culture according to any of claims 1-13 is infected or derived from the purified retrovirus according to any of claims 14-18, the antigen showing the following negative tests:

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the antigen or epitope,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the antigen or epitope,

D) in immunoassays, antibodies directed against HIV-I, and HIV-II do not bind to the antigen or epitope,

the antigen or epitope being obtainable by subjecting cell fragments and/or medium from a cell culture according to any of claims 1-13 or purified retrovirus according to any of claims 14-18 to gel electrophoresis, applying serum from a diagnosed multiple sclerosis patient to the resulting gel and visualizing bound antibody by means of labelled anti-human antibody, comparing the visualized gel bands with a similar preparation made using sera from a number of healthy persons, identifying the bands which are antibody-bound in the preparation using the serum from a diagnosed patient and which are not bound in the preparations using sera from the healthy persons, and isolating such bands containing the antigen or epitope from corresponding gel electrophoresis, and optionally extracting the antigen or epitope from the bands and purifying the antigen or epitope.

20. An antigen or epitope derived from, produced by, or induced by the retrovirus with which the cell culture according to any of claims 1-13 is infected or derived from the purified retrovirus according to any of claims 14-18, the antigen showing the following negative tests:

B) in immunofluorescence analysis, antibodies directed against HTLV-I antigens p19 and p24 do not bind to the antigen or epitope,

C) in immunofluorescence analysis, antibodies directed against the retroviruses MuLV, SSV-1 (p28), FeLV or RD114 do not bind to the antigen or epitope,

D) in immunoassays, antibodies directed against HIV-I or HIV-II do not bind to the antigen or epitope,

and the antigen showing the following positive test:

A) in Western blotting performed on retrovirus-containing material purified by sucrose gradient

purification or Triton X-114 temperature induced phase separation or purified by adsorption to antibody-conjugated microbeads, binding by the antibodies anti-rat HTLV-I gp46/gp21: 30g, 1e, 5a, 69b; and anti-mouse HTLV-I gp46/pg21: 46 to the virus envelope protein is detectable,

the antigen or epitope being obtainable by subjecting cell fragments and/or medium from a cell culture according to any of claims 1-13 or purified retrovirus according to any of claims 14-18 to gel electrophoresis, applying serum from a diagnosed multiple sclerosis patient to the resulting gel and visualizing bound antibody by means of labelled anti-human antibody, comparing the visualized gel bands with a similar preparation made using sera from a number of healthy persons, identifying the bands which are antibody-bound in the preparation using the serum from a diagnosed patient and which are not bound in the preparations using sera from the healthy persons, and isolating such bands containing the antigen or epitope from corresponding gel electrophoresis, and optionally extracting the antigen or epitope from the bands and purifying the antigen or epitope.

21. A diagnostic agent which comprises an antigen or epitope according to claim 19 or 20.

22. A diagnostic agent according to claim 21, wherein the antigen or epitope is provided with a label.

23. A diagnostic agent according to claim 22, wherein the label is selected from enzymes, fluorescent substances, radioactive isotopes and ligands such as biotin.

24. A diagnostic agent according to claim 23, wherein the antigen or epitope is coupled to a solid support directly or via a spacer.

25. An antibody which binds to an antigen or epitope according to claim 19 or 20.

26. An antibody according to claim 25, which is a monoclonal antibody.

27. An antibody according to claim 25, which is a polyclonal antibody.

28. An anti-idiotypic antibody which is directed against the site of an antibody according to claim 25 which is reactive with the epitope or the antigen according to claim 19 or 20.

29. A diagnostic agent which comprises an antibody according to any of claims 25-27 or an anti-idiotypic antibody according to claim 28.

30. A diagnostic agent according to claim 29, wherein the antibody is provided with a label.

31. A diagnostic agent according to claim 30, wherein the label is selected from enzymes, fluorescent substances, radioactive isotopes and ligands such as biotin.

32. A diagnostic agent according to claim 31, wherein the antibody is coupled to a solid support directly or via a spacer.

33. A nucleic acid having a nucleotide sequence which is distinct from sequences from known retroviruses and which when used as a probe on samples of body fluid, such as blood samples, or tissue samples from a number of healthy persons and samples of body fluid, such as blood samples, or tissue samples from diagnosed multiple sclerosis patients, respectively, detects nucleotide sequences from diagnosed multiple sclerosis patients, which can not be detected in samples from the healthy persons, the

nucleotide sequence being obtainable by isolating nucleic acids from the cell culture according to any of claims 1-13 or the purified retrovirus according to any of claims 14-18 and determining the sequence.

34. A nucleic acid according to claim 33 being obtainable by isolating nucleic acids from the cell culture according to any of claims 1-13 or the purified retrovirus according to any of claims 14-18 by the use of virus-specific nucleotide primers recognizing specific regions of the nucleotide sequences from the retrovirus.

35. A nucleic acid having a nucleotide sequence (S) obtainable by using a retrovirus-related nucleotide primer recognizing conserved regions of known retroviruses to obtain nucleotide sequences derived from the cell culture according to any of claims 1-13 or the purified retrovirus according to any of claims 14-18 by PCR, optionally sequencing the nucleic acids obtained to identify sequences which are distinct from sequences from known retroviruses, testing the nucleic acids obtained by PCR or the sequences identified by sequencing by using them as primers on blood samples from a number of healthy persons and blood samples from diagnosed multiple sclerosis patients, respectively, and selecting, as the nucleotide sequence (S), the nucleic acids or sequences which give rise to the attainment of nucleotide sequences from diagnosed multiple sclerosis patients in PCR, but do not give rise to attainment of nucleotide sequences from healthy persons, or using such nucleic acids or sequences as primers for an additional PCR obtainment of nucleic acids from the cell culture according to any of claims 1-21, performing the testing defined above using such second generation nucleic acids or sequences identified therein as primers and selecting, as the nucleotide sequence (S), the nucleic acids or sequences which give rise to the attainment of nucleotide sequences from diagnosed multiple sclerosis patients in PCR, but do

not give rise to attainment of nucleotide sequences from healthy persons.

36. A diagnostic agent comprising a nucleotide probe which is capable of detecting a nucleotide sequence according to any of claims 33-35.

37. A method of diagnosing multiple sclerosis, an early stage or a pre-stage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to any of claims 1-13, comprising subjecting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient to a PCR analysis in which the sample is contacted with a diagnostic agent according to claim 36 allowing any nucleotide sequence to be amplified followed by detection of any amplified target nucleotide sequence.

38. A method of <u>in vitro</u> diagnosing multiple sclerosis, an early stage or a pre-stage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to any of claims 1-13, optionally combined with a method for detection of an infection with another virus such as a herpes group virus, comprising contacting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent according claim 36 comprising a nucleic acid according to any of claims 33-35 and determining the presence of any identical or homologous nucleotide sequences in the sample.

39. A method of <u>in vitro</u> diagnosing multiple sclerosis, an early stage or a prestage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to any of claims 1-13, comprising contacting a sample of body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent according to any of claims 29-32, and

determining the presence of bound antibody from the sample.

40. A method of in vitro diagnosing multiple sclerosis or a prestage thereof, a latent infection or a subclinical infection with the retrovirus of the culture according to any of claims 1-13, comprising contacting a sample of a body fluid, such as a blood sample, or a tissue sample from a suspected multiple sclerosis patient with a diagnostic agent according to any of claims 21-24 and determining the presence of bound antigen from the sample.

41. A method for producing an antigen or epitope characteristic to the retrovirus, which comprises cultivating cells harbouring a plasmid which contains and is capable of expressing a nucleotide sequence according to any of claims 33-35 which codes for a polypeptide which has the properties of the antigen or epitope of claim 19 or 20.

42. A method for producing an antigen or epitope characteristic to the retrovirus, which comprises synthesizing a peptide having an amino acid sequence derived from the nucleotide sequence of any of claims 33-35.

43. A method for producing a antibody according to claim 25, which comprises immunizing an animal with an antigen or epitope according to claim 19 or 20 or an anti-idiotypic antibody according to claim 28 or an antigen or epitope produced by the method according to claim 41 or 42 to obtain cells producing an antibody specific for the antigen, and isolating the antibody from the animal or the cells.

44. A method according to claim 43 for producing a monoclonal antibody according to claim 26, which comprises

immunizing an animal with an antigen or epitope according to claim 19 or 20 or an anti-idiotypic antibody according to claim 28 or an antigen or epitope produced by the method according to claim 41 or 42 to obtain cells producing an antibody specific for the antigen, fusing the cells with cells of cell line capable of rendering the fused cells immortal, and selecting and cloning the resulting hybridoma cells producing the monoclonal antibody, or immortalizing an unfused cell line producing the monoclonal antibody,

followed by growing the cells in a medium to produce the monoclonal antibody, and harvesting the monoclonal antibody from the growth medium.

45. A method for obtaining a protective immunity in an animal, including a human being, against multiple sclerosis caused by the retrovirus of the culture according to any of claims 1-13, comprising administering, to the animal, an immunogenically effective amount of a vaccine against Epstein-Barr virus.

46. A method accroding to claim 45, in which the vaccine is live or Epstein-Barr virus administered to the mammal at a stage in the development of the mammal in which the disease caused by Epstein-Barr virus has a mild clinical cause and cannot induce active production of the retrovirus of the culture according to any of claims 1-13 to result in multiple sclerosis, an early stage or a pre-stage thereof.

47. A method according to claim 46 in which the mammal is a human, and the stage in which the administration is performed is the pre-puberty stage.

48. A method according to claim 47, in which the vaccine is attenuated Epstein-Barr virus or an immunogenic antigen characteristic to Epstein-Barr virus and eliciting the formation of antibodies against Epstein-Barr virus.

49. A method according to any of claims 45-48, in which the mammal to which the vaccine is administered is a mammal which has been shown to carry the retrovirus of the culture according to any of claims 1-13 by any of the methods according to any of claims 37-40.

AMENDED CLAIMS

[received by the International Bureau on 18 March 1993 (18.03.93);
original claim 50 amended;
remaining claims unchanged (1 page)]

50. A method for obtaining a protective immunity in an animal, including a human being, against multiple sclerosis caused by the retrovirus of the culture according to any of claims 1-13, comprising administering, to the animal, an immunogenically effective part of a cell culture according to any of claims 1-12, a purified retrovirus according to any of claims 13-18 or a fragment thereof, an antigen according to any of claims 19 or 20 or an anti-idiotypic antibody according to claim 28.

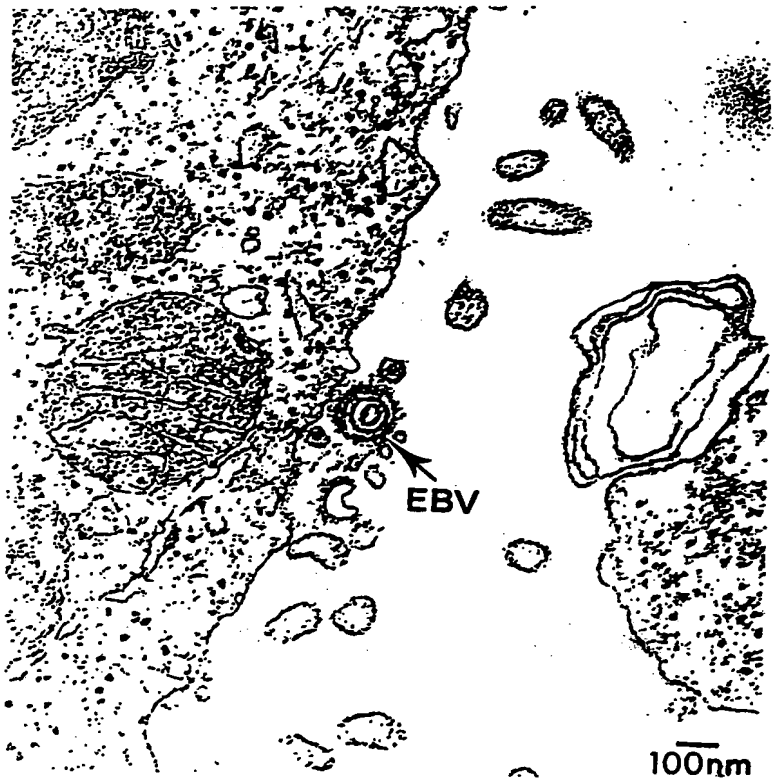

Fig. 1a

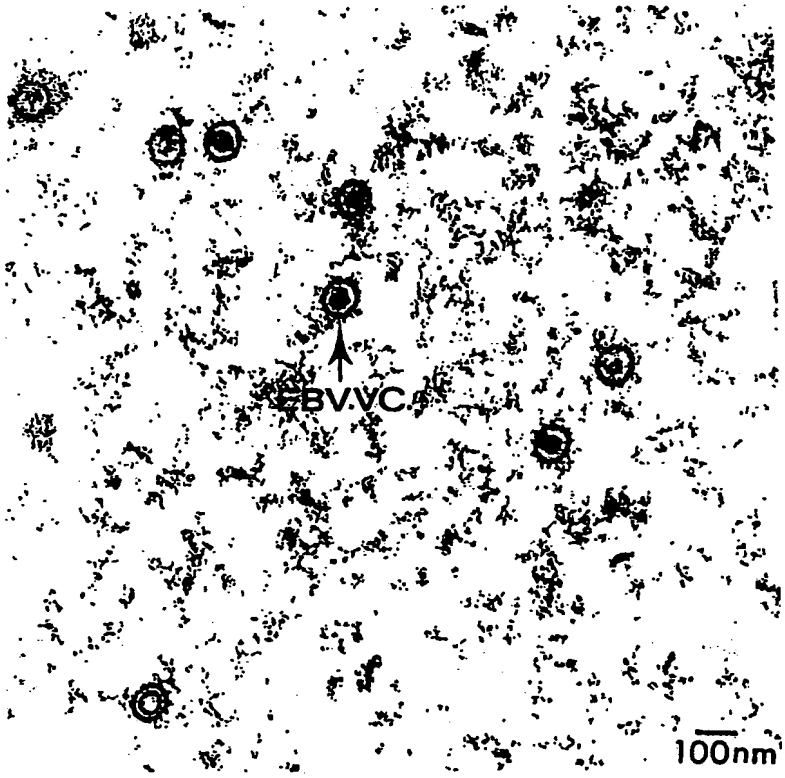

Fig. 1b

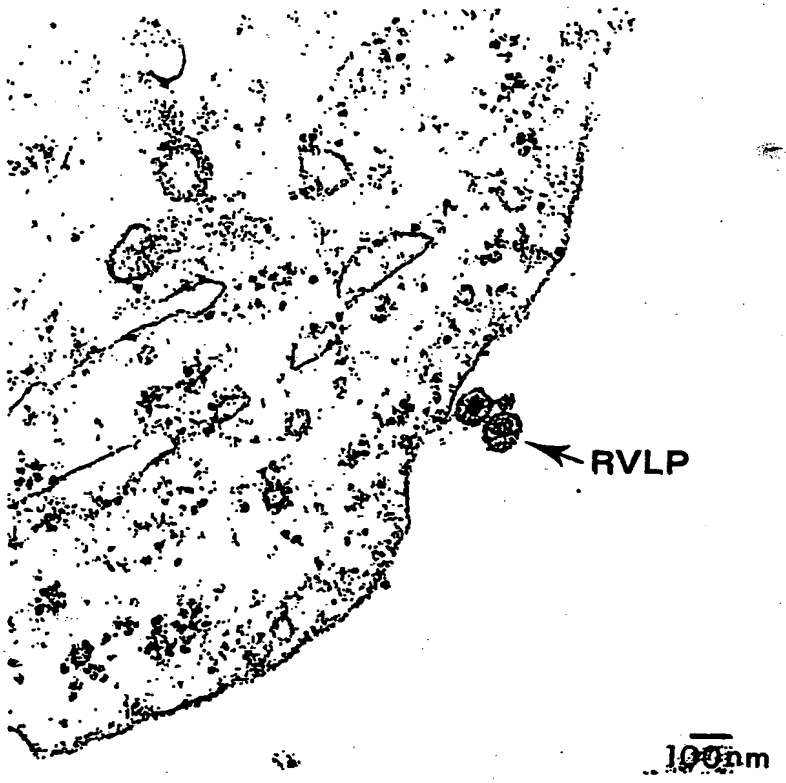

Fig. 1c

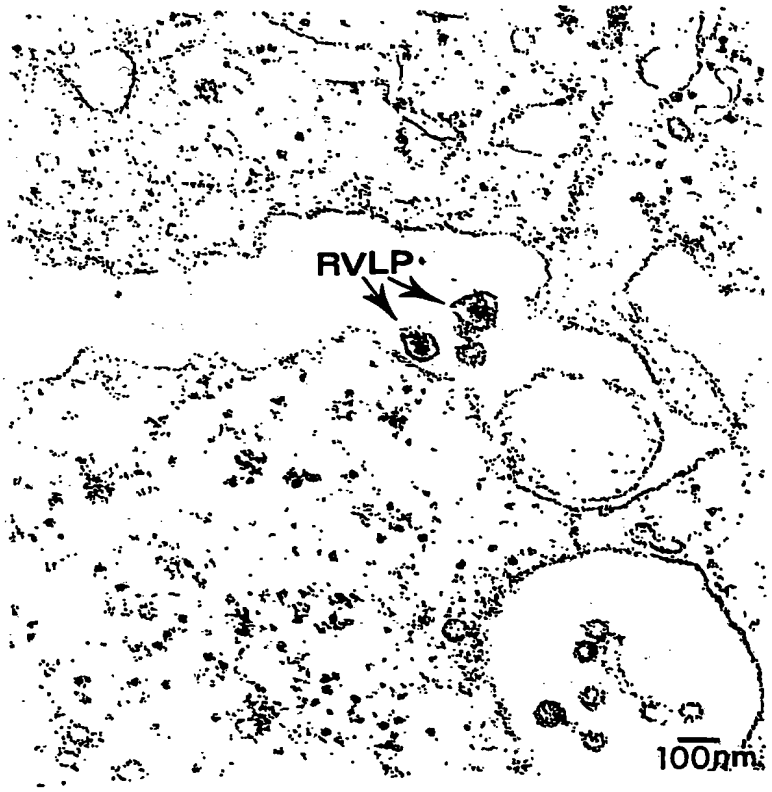

Fig. 1d

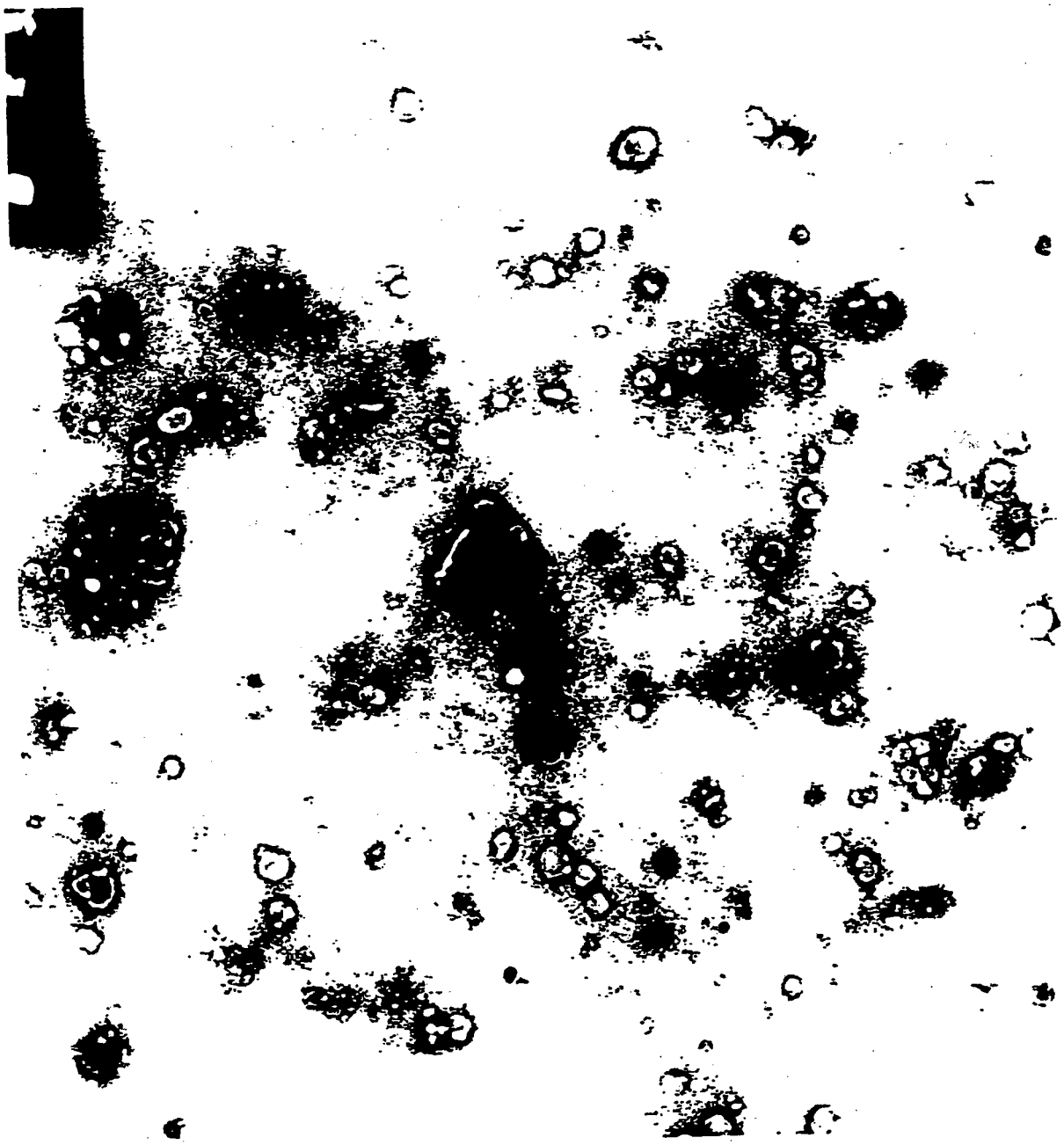

Fig. 2a

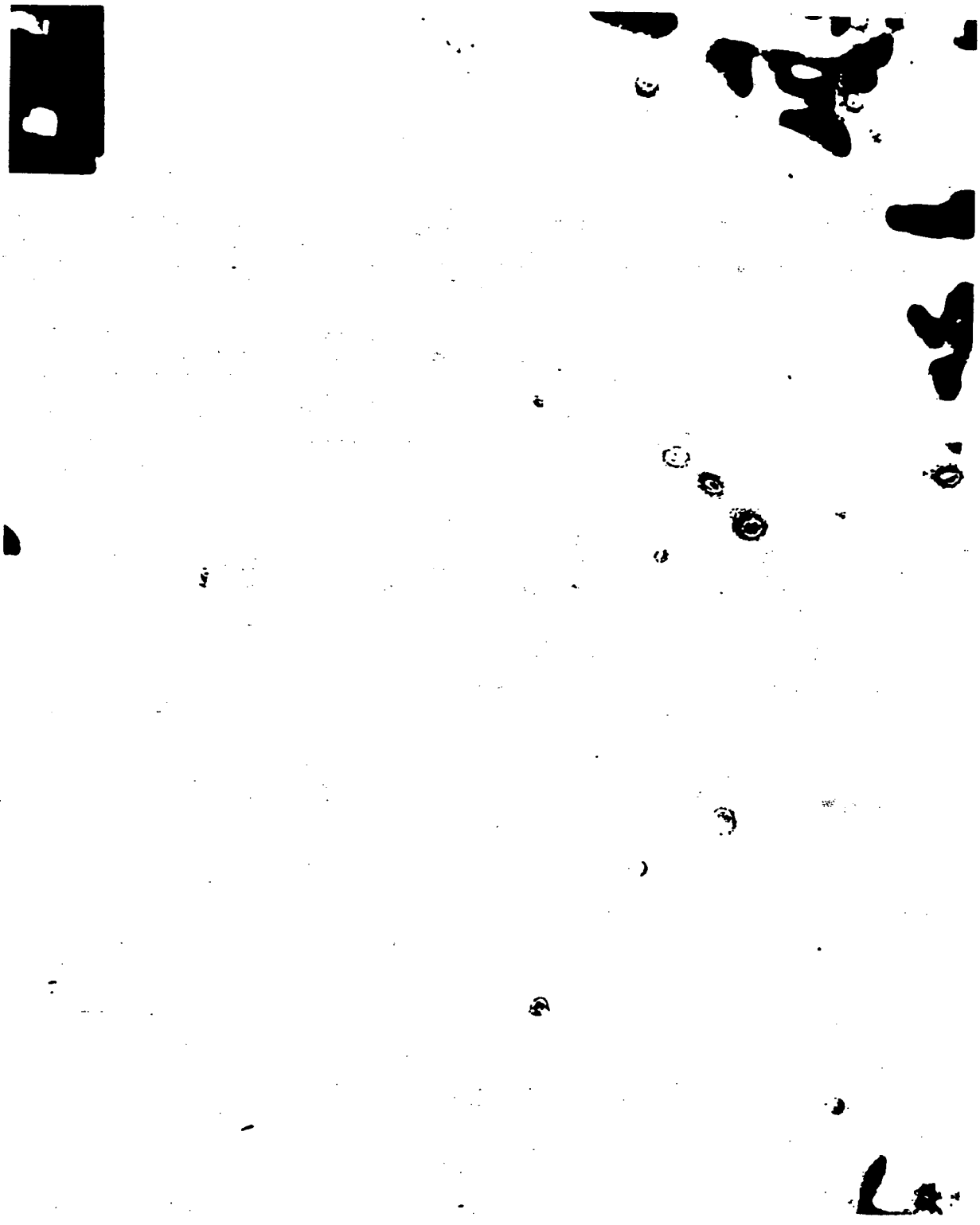

Fig. 2b

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00299

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 7/00, G 01 N 33/569, C 12 Q 1/70, A 61 K 39/21

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; C 12 Q; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	LANCET, Vol. 337, April 1991 Haar S. et al.: "Just another dubious virus in cells from a patient with multiple sclerosis?", see page 863 - page 864 --	1-6,8-11
X	LANCET, Vol. 337, April 1991 Peronn H. et al.: "Isolation of retrovirus from patients with multiple sclerosis", see page 862 - page 863 --	1-6,8-11,13-15,17-44
O,X	Journal of Neuroimmunology, vol. 38, (1992) Sven Haahr: Conference Report. Second International Symposium on Retrovirus in Multiple Sclerosis and Related Diseases, 26-28 August 1991, pages 163-174, see abstracts by Sven Haahr et al and Hervé Perron pages 172-173 and page 164, second paragraph. --	1-6,8-11,13-44

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th January 1993	18 JAN 1993
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl Olof Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	NATURE, Vol. 331, February 1988 Steven Jacobson et al.: "Isolation of an HTLV-1-like retrovirus from patients with tropical spastic paraparesis", see page 540 - page 543 --	1-44
T	Proc. Natl. Acad. Sci. USA, Vol. 89, March 1992 K. Banki et al: "Human T-cell lymphotropic virus (HTLV)-related endogenous sequence, HRES-1, encodes a 28-kDa protein: A possible autoantigen for HTLV-I gag-reactive autoantibodies", see page 1939 - page 1943 --	1-44
Y	Nucleic Acids Research, Vol. 17, No. 17, 1989 Andras Pearl et al: "Detection and cloning of new HTLV-related endogenous sequences in man", see page 6841 - page 6855 --	1-6,8-11,13-44
A	EP, A2, 0326395 (CITY OF HOPE) 2 August 1989, see the whole document --	1,13,33-38,42
A	EP, A2, 0384566 (THE WISTAR INSTITUTE) 29 August 1990, see the whole document --	1,13,33-38,42
A	EP, A2, 0222310 (THE WISTAR INSTITUTE) 20 May 1987, see the whole document --	1-44
A	WO, A1, 9208785 (THE UNIVERSITY COMPANY PTY. LTD.) 29 May 1992, see the whole document --	1,13

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 45-49, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00299

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 02/12/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0326395	89-08-02	AU-D-	2895289	89-08-03
EP-A2- 0384566	90-08-29	AU-D-	4878890	90-08-02
		CA-A-	2008731	90-07-27
		JP-A-	3139299	91-06-13
EP-A2- 0222310	87-05-20	AU-B-	598209	90-06-21
		AU-D-	6504386	87-05-21
		JP-A-	62194460	87-08-26
WO-A1- 9208785	92-05-29	NONE		